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Job-swapping could relieve academic stagnation

ACADEMICS in mid-career might consider exchanging posts for a period of up to five years. Those who scan the classified advertisement columns every week are only too aware that jobs for scientists in British universities are very scarce at present. As a result of low levels of retirement, disappointing recruitment of students into the sciences, and continued financial stringency which makes every new appointment subject to the closest scrutiny, there are no more academic jobs on the market in the late 1970s than there were in the early 1960s, before the great university expansion.

The plight of those at the bottom of the academic ladder has been well publicised over the past year or two — they are finding that if they are lucky they can subsist for a few years on postdoctoral fellowships before obtaining a permanent post, but without such fortune even highly capable young people may simply have to give up any aspirations to an academic career.

Less well publicised is the problem of the person in mid-career. Remarkably few academic positions are advertised at this level, because remarkably few academics are at present vacating their posts because remarkably few academic positions are advertised because Those jobs that are freed because their holders move to professorships are largely filled internally because almost every department is experiencing heavy pressure for promotion in the ranks. The result, as is well known, is stagnation as everyone holds tight to the job they have. And stagnation ultimately leads to demoralisation.

Dr Colin Clarke, of the School of Biological Sciences in the University of East Anglia puts forward a simple proposal. He writes:— “A simple, admittedly imperfect and partial solution to this problem would be that of direct academic exchange. Thus a lecturer in, say, genetics in University A might exchange his position, and possibly his house, with a similar person in University, or even Research Institute, B with great potential advantage and stimulation to both individuals involved and their departments. At its simplest this scheme could be largely informal and need not involve more expenditure than that of mutual visits and removal costs. House exchange, rather than house purchase, might avoid further financial problems. I suggest that for both academic and family reasons there would need to be a minimum period for such exchange, of perhaps 3 or 5 years. A permanent, or longer

term, exchange could then follow, but only if both partners and both departments were satisfied with the arrangement.

“This scheme has the advantage that it allows for the exchange of people whose scientific interests are not exactly matched, in some cases to the clear advantage of particular pairs of individuals or departments. Thus someone working in, let us say, applied plant genetics might exchange with another person whose interests are recombination in bacteria, depending on the existing interests and staffing of the two departments involved.

“The above scheme might operate via a personal advertisements section in *Nature*. Below is a sample advertisement, of the type envisaged, which I have written for myself. There will, of course, be objections to this scheme, not least perhaps from universities if the persons contemplating exchange are not on identical salaries. The present national academic situation is, however, so stagnant that solutions of the above type are highly desirable and their advantages to individuals and departments far outweigh administrative objections.

EXCHANGE SOUGHT

Reader in Microbiology, University of East Anglia, Norwich, research interests in Microbial Mutagenesis and Bacterial Morphogenesis, general interests in many aspects of General Microbiology and Microbial Genetics. Extensive teaching experience mainly Microbiology and Genetics. Exchange over 5-year, or longer, period for Sept 1982 onwards with like person in University or Research Institute, perhaps in Midlands or Scotland. Please contact Dr Colin Clarke, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ (Phone 0603-56161, extn. 2232). It may be possible to arrange a housing exchange involving 4-bedroom, detached house with garage and gas c.h. in Norwich.

Would it work? Dr Clarke will be able to tell us fairly soon. The record of the civil service, which tried out a similar mobility scheme a few years ago, is no encouragement. Interest in temporary exchanges was insufficient to warrant continuing the scheme after a year or two. But maybe academics are different. And maybe there is a growing awareness of the importance of sideways moves. If so, then *Nature* will be glad to support such a scheme. □



Carter faces new dilemmas over non-proliferation

In the last of three articles about US attempts to regulate the social effects of science and technology, **David Dickson** describes the problems raised by President Carter's policy for controlling the spread of nuclear weapons

ONE of the main issues in the US presidential elections next year — just as it was when Jimmy Carter faced Gerald Ford three years ago — is likely to be whether the US should revise its attitude towards the relationship between the spread of nuclear energy and the proliferation of nuclear weapons.

Three years ago Carter gained considerable political capital from his firm stand against both the reprocessing of spent fuel and the development of fast breeder reactors, arguing that if not properly controlled, both could increase the risks of nuclear weapons proliferation — by encouraging production of weapons-usable plutonium. A week before the election he castigated President Ford's initiatives in the same direction as "too little and too late".

Now it is President Carter's own policies that are on trial. In particular attention has focussed on the results of the Nuclear Non-Proliferation Act (NNPA) passed overwhelmingly by Congress last year, and whether the strategy embodied in this act — attempting to control weapons proliferation through abrogation of commercial reprocessing and unilateral restrictions on uranium supply — remains the most appropriate direction to take.

Some argue that the apparent failure of the US to achieve a significant consensus in support of this strategy at the international level indicates inherent flaws. They point in particular to the provisional conclusions of the International Nuclear Fuel Cycle Evaluation (INFCE), set up at Carter's suggestion in 1977 and due to report formally next February, which are expected to give little support for the US strategy.

Others argue, conversely, that the administration has not been strict enough in applying its restrictions. "Many of us feel that there has been a great deal of backtracking by the administration, and that this seems to be accelerating rather than slowing down" said one Congressional aide last week, pointing for example to delays in implementing certain aspects of the act and the administration's apparent leniency in accepting conditions for the retransfer of spent nuclear fuels for reprocessing.

Three aspects of the proliferation issue are likely to rekindle a public debate which has been relatively dormant since the signing of the NNPA last spring. The first is the failure of the US, despite cutting off economic and military aid, to dissuade Pakistan from what many believe to be its current attempts to develop nuclear weapons. "If Pakistan does explode a

nuclear device, then it could have the same galvanising effect as Three Mile Island did," predicts one observer.

The second aspect is the growing economic crisis of the US nuclear power industry. At present the industry is still meeting past orders; but domestic orders have virtually dried up, and export orders may be the only way to keep the industry alive. But these are now seriously hampered by US non-proliferation policy.

The third factor affecting the debate will be the occurrence of a number of international events focussing on the proliferation issue, including the domestic debate on a comprehensive test ban treaty (hopefully agreed in draft form by the US, USSR and UK in the near future), the review of the Non-Proliferation Treaty signed in 1970, and the outcome to the INFCE discussions.

INFCE: technical or economic?

Despite a relative lack of support for the thesis that the threat of proliferation requires of itself a reduced commitment to reprocessing and fast breeders (even though this may result *de facto* from technical and economic problems), US officials claim that the INFCE exercise has been a success in getting the link between plutonium production and the threat of proliferation firmly on the international agenda.

"INFCE has accomplished the largest part of what it was meant to do. As well as making a number of specific suggestions on way to go forward, it has been a process of international education, and despite difference between individual countries, there has been a considerable convergence over the past two years", says Dr Nye, one of the chief architects of the act and now Professor of Government at Harvard University.

However INFCE is unlikely to result in any major international shift towards the position so firmly enunciated by President Carter three years ago. (Indeed some suggest that the study may have been partly arranged to get the President off the hook). And despite the US administration's insistence that it was always intended as a technical exercise, and not a negotiating forum, scepticism of the US stance is based on the concern that technical exercises have been used as a front for political and economic interests.

Many European countries, for example, have accused the US of using its anti-reprocessing stand as a means of exploiting its market potential as a major supplier of

enriched uranium, and of making up for its lack of progress in fast breeder design.

Political distrust also lies behind the rejection of US non-proliferation policies by Third World countries such as India and Pakistan. These countries see attempts by the US to prevent their access to nuclear technology, on any grounds, as an illegitimate infringement of sovereignty.

Domestically US politicians show little embarrassment for arguing that the US should use its position of nuclear hegemony to both articulate and justify its strategy on proliferation policy. "Unfortunately, certain countries have refused to see the wisdom of our position. If this situation persists, they may force the US to discontinue further nuclear collaboration," Representative Jonathan Bingham, one of the staunchest supporters of the NNPA, said recently.

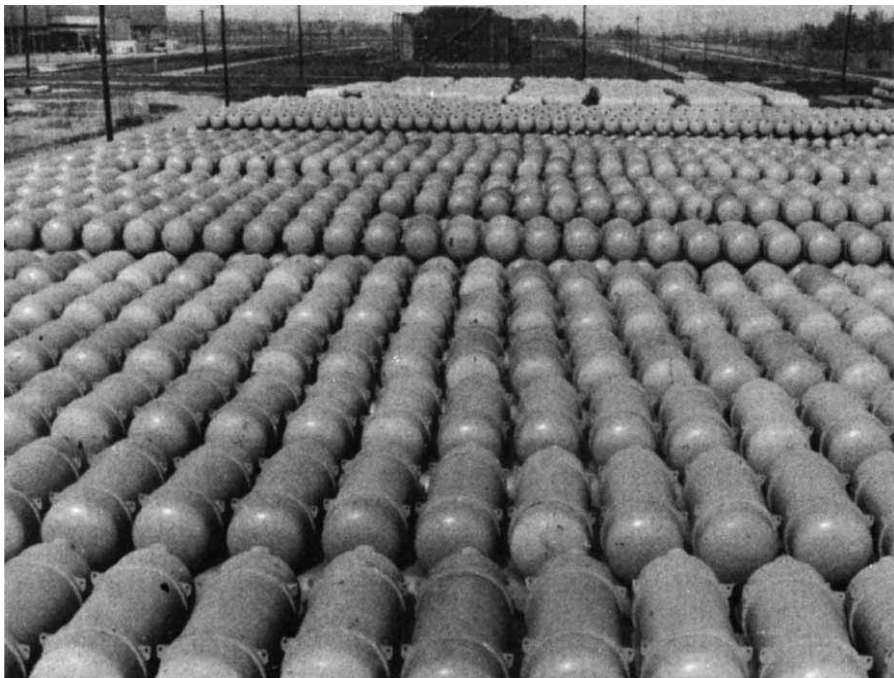
At the same time, administration officials are increasingly concerned that the US position on non-proliferation strategy may be undermining the US's hegemony on nuclear issues in favour of various European countries — and that this could have wide economic and political implications.

Last week, for example, the Senate rejected a motion to discontinue research on the liquid metal fast breeder reactor at Clinch River in Tennessee (a long-time target of President Carter's). The main arguments for rejection were couched no so much in terms of its technical and economic appropriateness, severely questioned by the administration, but on the argument that the US is several years behind Europe in fast reactor design, and that this alone was sufficient reason for pressing ahead with the project.

Given the distrust that exists both in Europe and in the Third World over the political implications of the US non-proliferation strategy, one of the few statements that can be made with certainty is that international consensus is still a long way off — and that reaching such a consensus will be one of the major diplomatic tasks of the 1980s.

One major source of contention during the INFCE discussions, for example, has been on reaching agreement on the extent of global uranium supplies. Those opposed to the urgent need for reprocessing have argued, in support of their position, that global supplies are plentiful; those supporting both reprocessing and fast breeders support a much lower estimate, adding weight to their position.

The resulting assessment of supplies is likely to be an ambiguous compromise between these two positions, with a range of possible estimates. "The vocabulary may be largely technical, but the messages and stakes are highly political, and as such INFCE presents a fascinating case study in the interaction of science and politics,"



Canisters of uranium-238 stockpiled at Paducah, Kentucky — the 'spent fuel' that can be converted to plutonium in a fast breeder

commented Peter Clausen of the Department of Energy's Office of Energy Research in a recent issue of *Arms Control Today*.

Domestically, the provisions of the Nuclear Non-Proliferation Act have placed new demands on the agencies required to see that they are carried out — in particular the Department of State, the Department of Energy, and the Nuclear Regulatory Commission.

The State Department, for example, has had to bear the brunt of external criticism of the administration's policies, particularly from countries with which it is actively seeking cooperation in other spheres; some critics claim that this has tempted the department to make compromises in carrying out its role — for example, in allowing reprocessing for the purposes of breeder research — that go considerably beyond Congress' original intentions.

The role of the Department of Energy, long an ally of the very industry which has been most affected by the NNPA, has also come under fire, critics claiming a conflict of interest between its functions of promoter and regulator of enriched uranium exports.

Finally several members of the Nuclear Regulatory Commission have expressed public disquiet about being asked to judge the adequacy of safeguards in countries which made requests for enriched uranium. This required the commission to play a direct foreign policy role for which it is ill-equipped.

"It is a major foreign policy action for an agency of the United States Government to go to a foreign country and say: we must inspect your facilities, we must inspect your system, before we will grant an export license. The NRC staff are not foreign

policy experts," Commissioner John F Ahearne said in an address "Does the Emperor Have any Clothes?" given in New Orleans three weeks ago.

Government agencies, however, are not the only ones having difficulty with grappling with the issues thrown up by the debate proliferation. There are also deep differences in strategy among environmental groups concerned with the dangers of nuclear power in all its forms.

Some argue that the prime need is to define the technical parameters of the licensing process in a way that minimises the environmental impact of nuclear power. "We have tried to get political considerations out of export licensing," says Jacob Scherr of the Natural Resources Defense Council (NRDC), arguing against Commissioner Ahearne that it is possible to define criteria for the adequacy of safeguards that are "technical and objective".

Others, however, argue the case against nuclear technology on the more explicitly political grounds of opposition to the social and economic interests which it supports. "The NNPA is more a mechanism for sharing the nuclear market than an attempt to curtain nuclear weapons in the developing countries; certainly it is looked upon by these countries very cynically as a market control mechanism," says Bob Alvarez of the Environmental Policy Centre (EPC).

Such different perspectives can lead to a considerable divergence in policy. A particular case is over whether or not the US should fulfil its commitment to provide storage facilities for fuel which has been used on foreign reactors. Groups such as the NRDC argue with the administration that providing such storage, coupled with a guarantee of providing enriched uranium fuels, will take the pressure off the need for

reprocessing facilities in foreign countries — and hence help reduce the chances of weapons proliferation.

In contrast, EPC and other nuclear groups argue that the US's provision of storage facilities would not only present a potential hazard to the US environment, but would also help encourage the spread of nuclear energy by getting other countries off the hook on the question of waste disposal. "Our philosophy is more concerned with restricting the spread of nuclear technology itself, rather than using a safeguard approach based on restricting the supply of nuclear materials," says Dr David Berrick of EPC.

Somewhat less predictably, the proliferation issue has also recently raised some difficult constitutional questions. These result from the recent publication of an article giving scientific and technical details of the construction of the hydrogen bomb, all claimed to be derived from public, non-classified information.

Finally there is the thorny question of how to deal with the Non-Proliferation Treaty when it comes up for review next year. At one level there is the question of the extent to which recent moves by the nuclear countries to restrict the export of "sensitive technologies" contravene Title IV of the NPT, which promises "the fullest possible exchange of equipment, materials and scientific and technological information for the peaceful uses of nuclear energy."

Perhaps more fundamental is the fact that, as it currently stands, the treaty codifies an inherent inequality between weapons states and non-weapons states. This condition that may have appeared appropriate when the treaty was negotiated in the late 1960s; but it fits uneasily with the demands made for a new international economic order, implying a form of North-South hegemony which many countries have demonstrated their unwillingness to accept by refusing to sign the treaty.

In the short-term, the Carter administration is now faced with the prospect of Pakistan exploding a nuclear device, and thus joining the "nuclear club" within the relatively near future. A state department study group was set up under the direction of Mr Smith late in August to discuss possible strategies by the US to prevent this, given that its current attempts to apply pressure through sanctions seem to have failed. And already some are suggesting that these sanctions should be stiffened.

In the long-run, there is the issue of whether a sufficient consensus — involving both developed and developing countries as partners in decision-making — can be forged to develop and apply an effective control regime; or whether increasing competition between both producers and consumers of nuclear power will be such as to limit the possibilities for multilateral action, shifting the focus back to bilateral actions. At this stage, nobody pretends to have all the answers. □

US adopts new cancer policy

NEW guidelines setting out a common framework for both the scientific assessment of potential carcinogens and the appropriate regulatory mechanisms were announced by the US Government last week. The guidelines, described as a "Government-wide cancer policy", were published by the regulatory council set up by President Carter last October to coordinate federal regulations in general. (See *Nature* 27 September 1979 p244).

On the scientific side, the council supports that view that animal tests are a valid method of determining whether a substance should be considered a potential carcinogen. It also says that a single animal test, if properly conducted, is sufficient to indicate "a potential risk to humans" for regulatory purposes — a view contested by the chemical industry in current debates over the proposals of the Occupational Safety and Health Administration to establish a "generic carcinogen policy".

Referring to claims that a single positive test is not enough, the council says that "although the agencies should attempt to obtain additional data, they should not

take the risk involved in waiting two to four years" to conduct further tests before taking action. At the same time, the council has gone some way to meeting industry's demands by caling on agencies to ensure that proposals for regulating carcinogens are accompanied by some form of risk assessment, including in particular determining how many people are exposed to the substance.

The council implies that economic considerations should be included, urging agencies to identify and consider the least costly and least disruptive course of action in planning cancer controls. It also says that agencies should consider "zero risk" bans in cases where the economic and social costs are considered minimal.

"This new policy will ensure that the various agencies will operate from the same scientific base in determining whether a compound is carcinogenic", Mr Doug Costle, chairman of the Regulatory Council and head of the Environmental Protection Agency, said in announcing the new guidelines.

David Dickson

School for scandal?

Soviet University admissions procedures are being circumvented by over-ambitious parents, *Pravda* alleged recently. The offenders, it claimed, are making use of their professional position or contacts to obtain their children a priority position on the placement lists.

Admission to Soviet Universities and other higher educational institutions is by competitive examination. However, even for those who have passed the examination, there may still not be enough places. Priority is therefore given to "front-rankers" from agriculture or industry, who are recommended and supported by their former place of work, to which it is assumed they will return.

This priority scheme is intended to provide Soviet production with the specialists it needs where and when it needs them. Modifications are made from time to time to meet specific demands. Thus the normal length of service needed for a "front-ranker's" place is two years. However, in April of this year, workers sponsored by the Ministry of Land Reclamation and Water Conservancy were exempted from any minimum length of service presumably due to pressing demands for graduates in that sector.

Although no less than four signatures and an official seal are needed on the "front-ranker's" sponsoring form, "crafty parents" says *Pravda*, are managing to equip their sons and daughters with false papers. Not surprisingly, those who graduate under such auspices are in no hurry to start working at the farms or enterprises which sponsored them. Not surprisingly, too, a

"Before you go in, stick this chicken under your arm and put some manure on your boots!"



number of the alleged "front rankers" have been abandoned in mid-course by their "sponsors" and have had to apply for state maintenance grants. Last July, the Central Committee of the Communist Party of the Soviet Union and the USSR Council of Ministers adopted a new decree on the "Further Development of Higher Education and Raising the Standard of Training of Specialist".

This provides for a new procedure of job allocation. By fixing a student's future job some two or three years before he or she graduates, the "output" of young scientists and technologists will, under the new system, become part of the normal Five Year Plan. Moreover, and particularly in the Far North and Siberia, measures are envisaged to retain the graduates in the area on a permanent basis.

Parents about to furnish their children with the certificates of Arctic lumberjacks would be well advised to think twice.

Vera Rich

Superpower clash over test ban technology

Delegates to the Geneva talks on a comprehensive test ban, currently nearing completion after almost two decades of negotiation, have reached an impasse over USSR demands that only Soviet equipment be used for the seismic monitoring on Soviet soil.

Until three months ago the US had assumed that the Soviet Union would permit the use of American detectors on its soil. Data from the detectors will be used to verify that seismic disturbances are the result of natural causes and not the underground testing of nuclear weapons banned under the terms of the treaty.

However in June members of a US Congressional delegation visiting Moscow were told by Soviet officials that they would only permit Russian equipment to be used for this purpose — a condition which, in the eyes of the US politicians, would make the treaty unacceptable.

"We feel that it is essential to use US equipment to verify Soviet compliance; and that this point is so central that we cannot allow the test ban talks to proceed unless this is resolved," an aide to Representative Jack Kemp (Rep, New York), said recently.

State Department officials in Washington have been playing down the significance of the USSR demands, claiming that they see them as essentially a negotiating tactic which will be dropped in return for some concession from the US side. They have already rejected a request from Mr Kemp to cancel a visit by Soviet scientists last month to study US seismic technology — a visit which Mr Kemp described as "scientific espionage".

Furthermore seismic experts in the US feel the USSR would have little to gain from insisting on the use of their own equipment, since the US already possesses suitable equipment that can be bought "off the shelf"; existing Russian equipment, it is claimed, lacks either the dynamic range or the precision of US seismic sensors.

Others involved in the negotiations, however, maintain that although relatively trivial, the issue is the sort which could seriously delay completion of the treaty, since no quick way out of the impasse can currently be seen. Any such delay could also effect the outcome of the second Non-proliferation Treaty Review Conference, which takes place next spring, and at which the non-nuclear powers will demand evidence from the nuclear powers of steps which have been taken to prevent escalation of the nuclear arms race.

Congressional aides said in Washington last week that they expect the issue of Soviet technology to be raised during hearings on progress towards the test ban treaty due to be held by the House Armed Services Committee later in the autumn. □

news in brief

"Employers need freedom": Mr Mark Carlisle, UK Secretary of State for Education and Science has refused to establish an enquiry into the problems of short term contract researchers. In a letter to the Association of Researchers in Medical Sciences (ARMS) the minister's private secretary transmitted Carlisle's sympathy for those on short term contracts and his reluctance to see "good research wasted because it had to be ended prematurely". But, in accordance with current Conservative philosophy, Carlisle affirmed the importance of the employer in economic development. "On the other hand, the effective development of research is liable to be hampered unless employers have sufficient freedom to manoeuvre." An ARMS spokesperson said the government's response was "dismal."

Piperno hearing postponed: Extradition hearings in Paris for Franco Piperno, the Italian physicist accused of anti-government activities (27 September) have been postponed until 27 October. In the meantime, the French civil liberties organisation CINEL (Centre d'initiative pour des nouveaux espaces de liberté) has raised questions about the prison conditions of Piperno and another detainee, Lanfranco Pace. In Italy 60 Milan lawyers have issued a statement opposing the extradition. The lawyer's statement accuses the Italian authorities of not wanting a true public inquiry but instead "to submit (Piperno and Pace) to a long preventative detention in special well known prisons."

India-USSR to collaborate on solar energy: An Indian team of scientists which visited USSR in July has signed an agreement to collaborate with the USSR on a programme of solar energy utilisation. The programme envisages the development and testing of selective surfaces and solar collectors, solar thermoelectric generators and an autonomous power station using a Stirling engine. The joint work will be carried out at Central Salt and Marine Chemicals Research Institute, Bhavnagar; National Physical Laboratory, New Delhi; Kryzhizhanovsky Power Engineering Institute, Moscow; and the Institute of Physical Studies, Tashkent. *From Zaka Imam, New Delhi.*

New Finnish nuclear incident: Following recent difficulties with the Soviet built Loviisa nuclear power station, a radioactive leak occurred at the Olkiluoto-1 station, which is of Swedish origin. The incident on 29 August took place when the station was working at 65% capacity. A pipe in the reactor water purification circuit burst, and some 5 m³ of radioactive water escaped on to the floor. All safety systems worked satisfactorily, and, according to the Teollisuuden Voima (Industrial Power) Company which owns the station, the leak was immediately isolated and the station shut-down. After tests by the Radiation Protection Institute, the station was allowed to recommence operation on 3 September. The shut-down had no effect on Finland's plans for nuclear energy expansion. While Olkiluoto-1 was still out of action, the Council of States granted an operating licence of Olkiluoto-2 (subject to final checks). This second set is expected to commence generating at the beginning of 1980 and to reach full capacity by the middle of the year.

More Windscale troubles: Thirty people had to be evacuated from a low level effluent treatment plant on 12 September when radiation alarms sounded in the early morning hours. Plutonium was part of the radioactive airborne release but the details on levels or amounts is not known. A British Nuclear Fuels spokesperson said that tests have shown "no signs of harmful contamination". The cause of the alarm and the source of the leak are the subject of an internal inquiry. In the meantime the inquiries into the fire in the decanning cave last July in which 8 workers suffered radiation exposure (26 July) and the sump leak last March that discharged 30,000 curies under a temporary storage building (26 April) are "still proceeding."

IEA launches International Energy Conservation Month: International Energy Conservation Month for the 20 members of the International Energy Agency began on Monday. To increase consumer awareness of the need for energy conservation, the IEA member states are organising 30 international conferences and exhibitions to take place during October in different cities throughout the world, as well as individual national events including local meetings, exhibitions, demonstrations and competitions for school children. In addition, two countries, the US and Japan, are using the month to launch new legislation on energy conservation. President Carter, for example, is expected to announce the enforcement of the 55 mph speed limit and the issue of a gas mileage guide.

Plant physiologist expected to be named head of new US research foundation: Dr Nyle Brady, currently director of the International Rice Research Institute in the Philippines, is expected to be nominated by President Carter as the director of the new Institute for Scientific and Technical Co-operation (ISTC), which will become the focus of US research efforts on issues directly related to the needs of developing countries. Two of Dr Brady's principal research interests have been the fundamental effects of lime in soil, and the physiology of the peanut plant (an interest which White House officials deny had any bearing on his appointment). Other research interests include the influence of fertiliser on the yield of rice, corn and coffee, and the influence of soil temperature on nutrient uptake.

Meanwhile the Senate appropriations subcommittee last week approved an allocation of \$19.75 million to the ISTC for the fiscal year beginning 1 October 1979, in addition to funds transferred from programmes currently under the Agency for International Development. However the institute's budget figure still has to be approved by the full Senate appropriations committee and by the Senate itself — at both of which stages it is expected to meet with opposition — before the institute can be assured that it will be allowed to start functioning.

US agrees to increase research cooperation with Japan: The United States and Japan have agreed to increase cooperation on scientific and technological research into non-energy fields, a move which follows an earlier agreement to cooperate on energy research. The broad framework for cooperation was agreed at a meeting in Tokyo last week between a US delegation headed by Dr Frank Press, director of the Office of Science and Technology Policy, and a Japanese delegation headed by Mr Moyazaki, deputy minister for foreign affairs.

Following summit talks held last May, a communique was issued stating that the two governments "will study seriously the prospects for cooperative efforts in areas of basic and applied research", adding that promotion of joint cooperation was important in building up a partnership between the two countries for the 1980s.

No details have yet been announced of specific areas for joint research activities. However possibilities include, outer space development, and environmental and health research.

Bombay magnetic observatory may move: The Colaba-Alibag Institute of Geomagnetism, with its 133-year-old record of continuous magnetic observation, may be moved if electrical noise from a nearby fertiliser complex disrupts its function. A campaign (*Nature* 15 February) to have the fertiliser complex at Thal-Vaishet resited has failed, in part because the state government of Marathashta opposed the move because of the loss of jobs resiting would have entailed.

A number of experiments on the effects of electromagnetic noise have so far proved inconclusive, however, and there is still the possibility that the observations will be unaffected by the plant's machinery.

Burying Britain's radioactive waste — the geological areas under investigation

OVER the next decade a major research effort is planned in the United Kingdom into the feasibility of the disposal of solidified, highly-radioactive, heat-emitting wastes to geological formations. Such research was called for in the Sixth Report of the Royal Commission on Environmental Pollution published in 1976¹ and accepted as one of three possible disposal options in the Government's White Paper² of 1977. These are disposal:

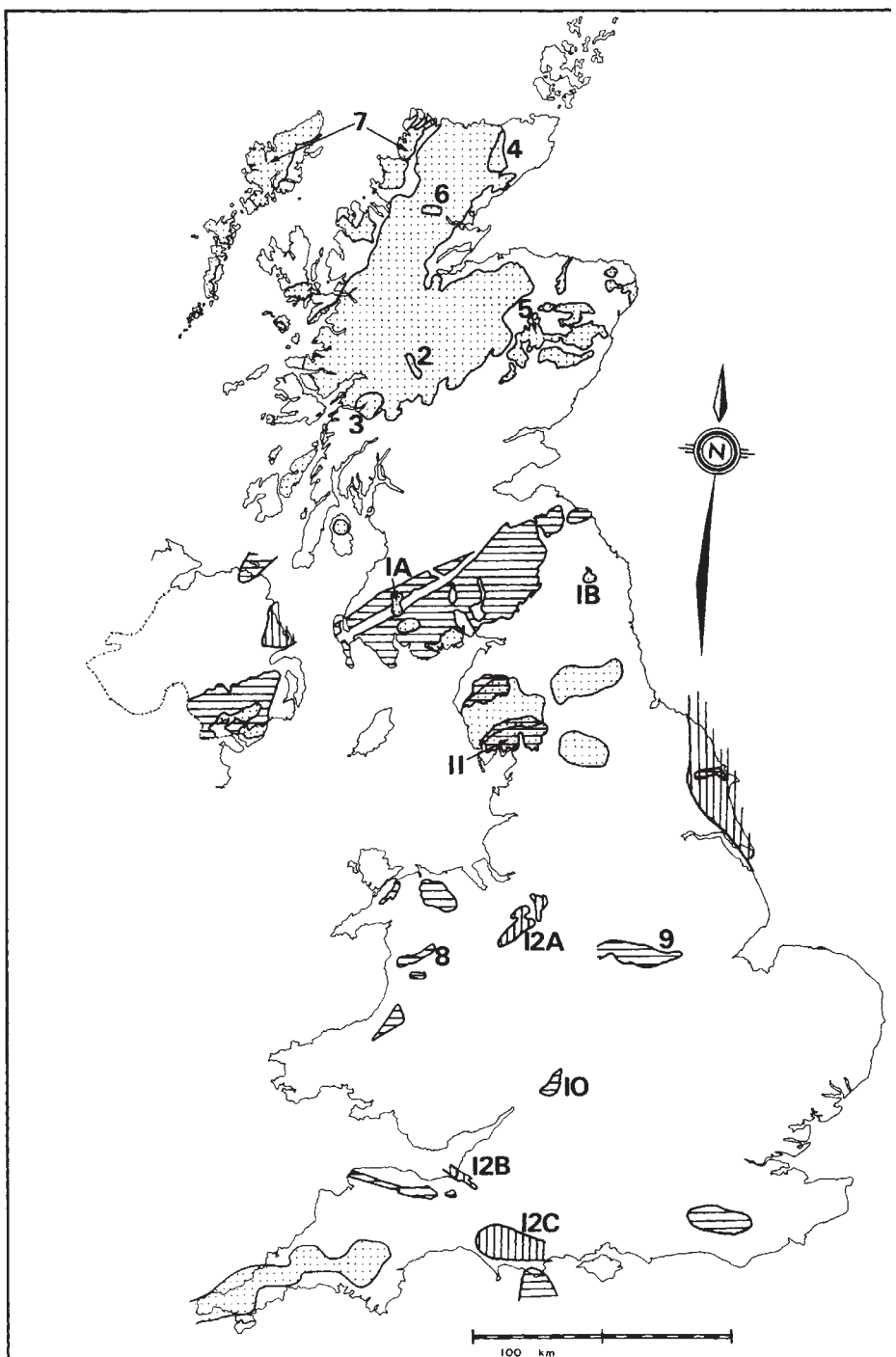
- on the bed of the deep ocean,
- into stable geological formations on land, and
- disposal under the bed of the ocean.

Parallel studies into all three options in relation to high-level wastes are being undertaken but the land option has progressed rather further than the oceanic programmes. The White Paper recognised that the Natural Environment Research

**By J D Mather, D A Greenwood
and P B Greenwood of the
Institute of Geological Sciences**

Council's Institutes of Geological and Oceanographic Sciences will play an important part in this research and relevant programmes are now under way at both Institutes. Criteria for use in the selection of areas under land which contain geological formations potentially suitable for such disposal in the United Kingdom were published in 1976 by a Working Party established by the Council's Institute of Geological Sciences³. A recent comparison of the criteria with those published elsewhere has indicated a high degree of international agreement, so that no significant revisions are necessary⁴.

The principal objective of disposal of these solidified wastes into geological formations is to ensure that they are placed in a situation where the radionuclides cannot be returned to the biosphere in amounts constituting a biological hazard. In reality that means placing them in a repository constructed at depth in a geological formation or rock mass able to withstand the effects of decay heat and radiolysis and sufficiently free from circulating groundwater to ensure their containment^{5,6}. The programme is planned in a series of stages of which the present proposals relate to the exploration



Map showing the distribution of rocks thought to meet the selection criteria and of those areas which have been selected for feasibility studies. Crystalline igneous and metamorphic rocks are stippled, argillaceous rocks are shown with horizontal lines and evaporites with vertical lines. The crystalline rocks include the postulated subsurface outcrops of the granites of northern and south-western England. No rock types meeting the criteria occur in the Shetland Islands.

phase, during which no radioactive wastes will be used. Some sections of the UK national research programmes are being mounted jointly with the Commission of the European Communities.

The application of the geological criteria results in the recognition of 127 different areas containing potentially suitable host rocks. These range in area from less than 5 km² to around 6,000 km², the mean being around 260 km². Of these areas, 104 contain crystalline igneous or metamorphic rocks, 17 argillaceous rocks (fine grained sediments consisting

principally of clay minerals) and six evaporites, including hybrid evaporite/clastic sequences. The distribution of the areas involved is shown in the map above and covers nearly 39,000 km², approximately 16% of the land area of the UK.

In order to select representative areas for feasibility studies a further desk study was undertaken using as primary factors the geological environment and the evolutionary history of the rocks concerned, so far as they are known. The groups thus obtained were then further

Areas for further study

Crystalline rocks

1. Caledonian granites intruded into (1A) Lower Palaeozoic sediments or (1B) volcanic rocks. The Loch Doon Granite (Intrusion 1A) is a zoned body with an outer unit of biotite tonalite merging progressively into a central unit of biotite granite. It is intruded into Ordovician sediments of various types, predominantly greywackes, but with significant developments of black shales and cherts and has a sharp contact with an extensive thermal aureole. The Cheviot Granite (Intrusion 1B) varies in composition from a pyroxene granite to a pyroxene-free granophyre, is intruded into andesite lavas and has a faulted brecciated contact.
2. Caledonian granite forcefully intruded into Pre-Cambrian metamorphic rocks. The Ossian Granite is a coarse hornblende-biotite tonalite varying to granodiorite towards the outer part. A number of SW-NE trending fractures cross the granite which is intruded into metamorphosed sediments belonging mainly to the Pre-Cambrian Moine Supergroup. No thermal aureole is developed but the intrusion margin is generally intimately welded to the country rock by the development of contact migmatites.
3. Caledonian granite permissively intruded into Pre-Cambrian metamorphic rocks. The Etive Complex is a ring complex composed of 4 successive granite intrusions emplaced as a result of progressive cauldron subsidence. The innermost unit known as the Starav Granite is a quartz adamellite which has a simple, near-vertical contact and is free of dykes.
4. Granitic complex in the core of a regional migmatite zone. The Strath Halladale Granite occupies the core of an injection complex within metasediments of the Moine

Supergroup. The injected material is dominantly biotite granite of variable grain size which occurs as veins or concordant sheets within the psammitic host.

5. Caledonian basic intrusive. The Morven/Cabrach Intrusion is mainly composed of norites with associated gabbros and diorites. It is steep-sided with steeper dips on the west than on the east, implying an increase in width at depth. Much of the intrusion is extensively altered with pyroxene replaced by amphibole.

6. Non-migmatized Pre-cambrian Moine Metasediments. The rocks of the Moine Supergroup consist of thick metamorphosed arenaceous and argillaceous sediments with some intermediate types. The area around Inveroykell is being considered within a region of massive to flaggy quartzo-feldspathic psammi of intermediate metamorphic grade and relative lack of structural complexity.

7. Pre-Cambrian Lewisian basement rocks. The Lewisian includes relics of several orogenic cycles covering a time span of at least 2,800 my to the latest reworking at 1,600 my. The major sub-division is into two time-based complexes, the older Scourian and the younger Laxfordian. The Laxfordian rocks are dominantly grey quartzo-feldspathic and biotite hornblende gneisses with intrusive sheets and veins of granite and pegmatite. The gneisses have undergone thorough re-working resulting in rock units that are relatively homogeneous compared with much of the rest of the Lewisian basement and appear the most suitable as a disposal medium. Such rocks occur in North West Scotland, between Loch Laxford and Cape Wrath and in the Outer Isles, but so far no specific research area has been identified.

Argillaceous and evaporite formations

8. A series of gently folded Ordovician and Silurian rocks in North Wales dominated by argillaceous types totalling some 4,000 m in thickness. The rocks are well exposed at the surface and dip consistently to the south-east at an angle of about 45°.
9. A sequence of turbiditic mudstones with interbedded siltstones and limestones of Lower Carboniferous age. The rocks occur within a fault bonded trough known as the Widmerpool Gulf and a minimum thickness of 1,000 m of argillaceous rocks is available. These rocks constitute a good example of the effective compromise desirable in argillaceous rocks — a thick formation that has been dewatered and developed a mature clay mineralogy by deep burial, without the attendant development of structural complexity.
10. A mudstone dominated sequence of Triassic rocks attaining thicknesses just in excess of 500 m in the central part of the

Worcester Basin. These occur within the evaporite-free part of the major depositional basin with a cover of up to 500 m of mainly argillaceous rocks of Jurassic age.

11. A series of Silurian mudstones and greywackes in Southern Cumbria with an overall thickness of around 5,000 m. These rocks are folded on both a large and small scale and are cleaved to a lesser or greater extent depending on lithological variations.

12. Hybrid evaporite/mudstone sequences within Permo-Triassic sediments developed in the Cheshire (12A), Somerset (12B) and Wessex Basins (12C). These are all major depositional basins where the rocks of interest are in excess of 600 m in thickness and are generally overlain by other argillaceous formations. In all these areas examination of the cyclicity of the hybrid sequence from the point of view of waste containment is a particularly important aspect.

divided on the basis of compositional differences, degree of consolidation (for argillaceous rocks), structural complexity and other aspects to produce a tentative order of preference based entirely on geological factors. It must be emphasised that this order of preference is necessarily based on subjective judgements; if the data were available for an objective analysis there would be no need for the present research programme.

As the programme advances and more data become available it can be expected that some areas will be rejected and others

introduced. It should also be noted that the areas selected relate to high-level wastes and that other formations in additional areas may be relevant to the disposal of intermediate and low-level wastes.

Crystalline rocks

Using the order of preference, 23 crystalline rock areas were selected for more detailed investigations, including reconnaissance field visits where possible. These 23 areas included a wide range of geological environments test the validity of the approach on which the grouping had

been based. A combination of those studies and selection based on the non-geological factors of land ownership and access to drilling sites resulted in selection of eight areas within seven different geological environments for detailed research into the feasibility of geological disposal of high level wastes into crystalline metamorphic and igneous rocks. Detailed analysis is required to relate the primary factor, groundwater flow, to rock mass and rock material parameters, such as mineralogy and petrology, joint frequency, attitude and openness, and regional stresses. A data base can then be considered so that the most appropriate crystalline environments for consideration as repositories can be defined.

The geological environments chosen for the future feasibility studies, and the individual areas concerned, are shown in the map and a brief description is given alongside.

Before work proceeds at any of the prospective research sites applications have to be submitted under the planning regulations for permission to drill investigatory boreholes. So far such applications have been submitted for sites within the Loch Doon Granite (Area 1A), the Cheviot Granite (Area 1B) and the Strath Halladale Granite (Area 4). Only the latter has been granted and the others are at present the subject of appeals. The research site within the Strath Halladale Granite occupies some 100 km² within the Caithness District and is peat and drift covered with only limited exposure of bedrock.

A preliminary drilling programme has been undertaken to confirm the limited geological information previously available and three boreholes have been drilled to 300 m, together with 24 boreholes to some 40 m. The data obtained from the boreholes complemented by field mapping, surface and borehole geophysics and detailed hydrogeological measurements are being used in a preliminary review of the containment characteristics of this particular geological environment. Similar studies are planned for the other research sites in the crystalline, argillaceous and evaporitic host rocks.

Argillaceous rocks and evaporites

The approach used in the studies of the feasibility of geological disposal to argillaceous rocks and evaporites is broadly comparable to that used for the crystalline rocks and total of seven preferred areas have been selected. The geological factor particularly relevant to the selection of these areas is the predictability of their mass characteristics. Impersistent developments of minor lithological variations may not pose problems but the presence of, for instance, a thin calcareous sandstone within an otherwise uniform greywacke/siltstone sequence, could be of major significance

from the viewpoint of waste containment.

A useful property of plastic clays is their potential capacity for self-sealing. With progressive compaction and burial this property will be lost but will be countered by an increase in thermal stability as a result of the expulsion on non-structurally held water. As these changes are mutually exclusive an intermediate compromise has generally been sought involving for instance selection of areas containing uncleaved mudstones.

Although salt domes occur beneath the UK Continental Shelf they appear to be absent beneath the land mass and the evaporite formations available are all bedded deposits. These generally occur within thicker argillaceous formations and are hybrid sequences containing interbedded evaporites and mudstones and/or siltstones.

The areas containing argillaceous and evaporite formations which have been selected provisionally for more detailed study are also shown in the figure and briefly described in the box.

It is hoped to mount studies to examine the feasibility of geological disposal to argillaceous rocks at sites in each of areas 8-11 and at one site within areas 12A-12C for preliminary studies on the hybrid evaporite/mudstone sequences. Work has not advanced as far as with the crystalline rock studies and at the present time no research sites have been identified and no applications for permission to drill boreholes have been submitted.

Conclusions

The feasibility of the disposal of high-level radioactive wastes within the geological framework of the United Kingdom still remains to be demonstrated. The immediate need is for comprehensive site-specific field data from a range of potentially appropriate geological environments both to test the criteria on which these environments were chosen and to provide a data base from which potential disposal sites may be identified. The feasibility studies suggested represent a major step towards these objectives and need to be initiated as a matter of urgency.

Sun-soaked but blind to solar power

ON the counters of the Caixa General de Depositos, the largest government bank in Portugal, bright orange folders decorated with a smiling Sun attract everyone's attention. They announce credit facilities for equipping private homes with solar water heaters. Up to £500 loans can be obtained at 10% interest over 10 years, an extraordinary bargain in a country where inflation reaches 50% a year and interest rates on short term deposits are 20%. "The response, however, has been minuscule" says a bank official. "There is no echo of interest in the public; people just don't know what it is."

The first installation financed by the Caixa was hastily inaugurated on June 23, a Saturday christened Sun Day, in a village 20 miles south of Lisbon. Here a successful architect has equipped his new country home with three solar panels in the garden. This 300 litre system cost £800. The installer, Mr Falconer, is a British subject and one of the two pioneers of solar energy installations in Portugal. He told *Nature* "the bank wanted publicity, but the house was still not finished at the time of the inauguration; so we connected a plastic hose to the plates to prove to the press that they heated the water." Falconer's firm has been importing MIROMIT plates — selective coating plates developed by the Tabor group — from Israel since 1961.

The other solar energy pioneer is Mr Neves da Silva, who obtained a Portuguese patent for a simple painted flat solar collector in the early 1960s. He is director of PRETEC, a mechanical construction firm, and he first attempted to build his collectors from national materials. "We tried to use cork, Portugal's main export product, as an insulator, but the price was too high; so we have to use glass wool like everyone else."

Both men are enthusiastic practical engineers. They have believed in solar energy for the past twenty years in spite of general indifference and incredulity. "In the sixties I had to deal with an engineer who stayed up all night hoping to catch me heating up the water in the reserve tank with an electric resistor. That was the only way he could imagine I could get hot water during the day from my system" recalls Mr Falconer.

"In the early days solar energy was no more than a toy for the very rich. Today it may still be mainly 'to show to the English', as the Portuguese saying goes." Indeed, a stone's throw from the casino in the resort town of Estoril on the fashionable Costa do Sol stands a huge mansion which is, according to its owner, the only solar equipped house and swimming pool in

Portugal. Among the bougainvillea trees and parterres of geraniums are interspersed rows of solar collectors; the mansion itself displays several striking black overhangs on the south side. "My pool is heated up by 24 panels; my family has used it 90 days per year since 1961" says Virgilio Gomes Borges, one of the brothers of the Borges Brothers Bank. "I use 32 panels in the sanitary water circuit; additional heat storage is provided by the electrical company's reduced night rates."

Using the Sun's light remains an elitist activity haphazardly conducted in the midst of abundant talk about energy crisis on the one hand and Portugal's exceptionally favourable climatic situation on the other. Tourism officials claim 244 sunny days per year. A more objective but no less encouraging way of rating Portugal for its potential is through its atmospheric transmission ratio — of the energy received on a horizontal surface on the Earth to the energy received on an equal surface at the top of the atmosphere, averaged over a year. According to Dr Collares Pereira, the only Portuguese physicist with a PhD in solar energy work, that ratio varies between 0.3 for high latitude rainy climates and 0.7 for clear desert locations like Albuquerque in New Mexico. Lisbon's ratio is 0.65 (and the south of Portugal is considerably sunnier) while New York and New Jersey, at the same latitude, hardly reach 0.5.

The local capacity for building solar plates has improved over the years. Mr Neves da Silva remarks that back in 1962 he operated as "an artisan", but today PRETEC would be capable of producing 100 collectors per year. What is missing, however, are serious orders and some planning and stimulation on the part of the government. At the opening session of a Franco-Portuguese seminar on solar energy last March, Mr Sidonio Pais, General Director for Energy of the Ministry of Industry, declared that it was impossible for him to be specific about solar energy developments at the moment because "our government does not have yet an energy policy".

At the same seminar, the Secretary of State for Higher Education praised the ecological advantages of solar energy but spelled out no plans for the future.

In the private sector several religious schools have put up collectors to heat up sanitary water. "It is quite well adapted to the kind of financing that this type of institution can do" says Neves da Silva who built the systems, "religious groups can easily raise money once-off for such a project, and solar heating does not give rise to monthly costs afterwards."

For the future the Ministry of Health has in its files a construction program for 27 health centres throughout the country, each equipped with 75 solar panels for sanitary water heating. It should come up for bidding soon.

Maurice Bazin

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'Those who attack nuclear energy show a cynical denial of human ingenuity'

THREE Mile Island confirmed what many of us in the nuclear enterprise had suspected — that the "China Syndrome", rather than proliferation or even waste disposal, would ultimately decide the public's acceptance of nuclear energy. Nor is this unreasonable. Proliferation is an issue far from most people's perception; more than that, it faults nuclear power by association, not by intrinsic connection. As for waste disposal, despite the violent arguments, the nuclear community could always point to plausible technical solutions for wastes (one must remember that 800 years after wastes are created, their toxicity has fallen below that of the original uranium from which the wastes were created). But for the 15 billion curies contained in a 1000 megawatt reactor, we could never say that the chance of a serious accidental release was zero. Three Mile Island carried this message directly to the public; the public must decide whether it can accept this risk. Chancellor Schmidt's recent call for an international review of safety as a sequel to the International Nuclear Fuel Cycle Evaluation study of proliferation resistance is therefore most appropriate.

The fact that deaths caused by nuclear energy, are on average, considerably fewer than those estimated for most energy sources (including many solar sources if they are backed up by non-solar systems), seems to me rather beside the point. Risk as perceived by the public is not simply the product of probability and consequences — the first moment of the probability distribution of consequences. The very high moments of the distribution — very low probability high consequence events that contaminate large stretches of land — are probably more important. Thus a most important technical fix for nuclear energy would be a means of minimising the amount of land that conceivably could be contaminated in the worst possible accident. Siting reactors underground could achieve this, though at considerable expense. The recently published report on underground siting to the California Energy Commission concludes that comparable improvements can be achieved at much lower cost with more elaborate containment systems.

Three Mile Island suggests that even an accident that imposed an average additional radiation burden 1% of the natural background on those within 20 miles is unacceptable. For nuclear energy to survive, we must reduce the probability of any serious malfunction much below the 1 in 20,000 per reactor-year estimated in the Rasmussen report, as well as reducing any possible consequences.

Can we fix nuclear energy? I believe we can, but it will require important insti-

tutional changes as well as better technology. I am therefore most enthusiastic about the plan announced earlier this year by the Tennessee Valley Authority, the largest electrical generating entity in the United States, in response to Three Mile Island. The main elements of the TVA plan are:

- the establishment of a separate operating entity with the Office of Power that is responsible only for nuclear generation;
- the establishment of an independent Nuclear Safety Review Board that reports directly to the Board of Directors of TVA;

Alvin Weinberg (right) explains why, despite the Three Mile Island accident, he is still a believer in nuclear energy



- the professionalisation of the cadre operating nuclear reactors — operators will receive training equivalent to a bachelor's degree, and salaries high enough to attract and keep the best, even though this places them at an advantage as compared to operators of fossil fuel or hydroelectric plants;
- more extensive plans for evacuation, and for keeping the public informed on the status of its nuclear plants.

In addition to these institutional changes, TVA is, on its own, proposing a number of technical improvements for its reactors that go beyond what the Nuclear Regulatory Commission requires. TVA, being the largest American utility, is well equipped to analyse and then implement safety measures that a smaller utility might not be in position either to recognise or to pay for. It is to TVA's credit that one of its safety engineers, C. Michelson, had visualised essentially the Three Mile Island sequence a year before it actually happened, and had called the attention of Babcock and Wilcox, (the nuclear steam suppliers) and the Nuclear Regulatory Commission to the deficiency.

Although not mentioned in their plan, I would hope that TVA will confine future reactors to its existing seven sites rather than allowing nuclear sites to proliferate. The sites would be expected eventually to develop into powerful nuclear centres. They would be invested with a commitment of permanence: spent fuel elements, low level wastes, and decommissioned reactors could be stored for a long time on them.

After 100 years the high level wastes would be producing little heat and the low level wastes and reactors, though not innocuous, would be much easier to handle.

The TVA plan, together with confinement of the nuclear enterprise essentially to existing sites, seems to me to constitute an acceptable nuclear energy system. TVA, being large, self-contained, and able to set its own rates, is better able to implement such a policy than are most of the private utilities. But I believe the private utilities ought to move strongly in this direction — by establishing nuclear generating consortia, perhaps like the Yankee Atomic Electric Company, in each of the nine regional Reliability Councils; or possibly by assigning generation of all nuclear energy in a region to a single utility that has the strength to take on this responsibility. Such a nuclear generating company, from the president down to the operators, would think, act, and know the nuclear business, with all its responsibilities and hazards.

Will an aggressive acceptance by the US, or even the whole world, of some version of the TVA plan plus confined siting and technical fixes, be enough to tide nuclear energy over the present disaffection? I certainly hope so. The TVA plan, with its standby mobilisation of experts to respond to any contingency, ought to give fullest opportunity for the exercise of human ingenuity. The serious nuclear accidents, though they were precipitated by human error, were contained by human ingenuity. At Three Mile Island, shutting off the high pressure injection pumps was perhaps the key operator error; but only 12 curies of ¹³¹I were released — some 2000 times less than was released at Windscale in 1958. A key manoeuvre in preventing more iodine from escaping was the replacement of the iodine filters a few days after the incident began. This was carried out faultlessly by experts from various nuclear installations.

I consider myself to be a not uncritical advocate of nuclear energy: an advocate because I believe we need nuclear energy and the alternatives to it are faulted; not uncritical because I have been close to the technology for enough years to recognise its deficiencies. Despite Three Mile Island, I remain an advocate. Those who wish to extirpate nuclear energy base their position on an unquestioning acceptance of human fallibility and a cynical denial of human ingenuity. The two major accidents in the United States, Browns Ferry and Three Mile Island, suggest to me that this is an unjustifiably pessimistic assessment of the human condition. Rather, as the TVA Plan reveals, there is much room for creating institutions that will encourage ingenuity, and on which we can base an acceptable nuclear energy. □

correspondence

'Beast and Man'

SIR, — I have only just seen Professor Stuart Sutherland's very interesting review of my book *Beast and Man* (28 June page 83.) May I say something in reply to the difficulties he raises?

He says that the main aim of my book "seems to be to establish a secure basis for ethics" and that, like "most previous attempts to establish a secure basis for an ethical system", it fails. (The italics are mine). Two projects might be meant, of which the first is certainly not mine —

● A "secure basis for an ethical system" would be a justification of one particular set of moral views against others. This would itself be a piece of ethical reasoning.

● A "basis for ethics" by contrast seems to mean something much more general — a justification or explanation, from outside, of the whole range of thought which can be called ethical or moral. Confused people often run these two enterprises together, supposing that the second will do the work of the first. I think this is what Professor Sutherland objects to, and I share his objection. There can be no moral justification of morality as a whole any more than there can be a mathematical justification of mathematics as a whole; neither from outside nor from inside these areas of thought would such an enterprise make sense, nor is it needed. In the case of morality, the notion that it is needed, and is lacking, stems largely from Nietzsche's ambiguous talk of 'immoralism'. Nietzsche often spoke of this as a comprehensive attack on the very possibility of morality. But in fact his campaign was an intelligently limited one, directed against certain particular human weaknesses and particularly against contemporary bourgeois notions. It was conducted in entirely moral terms, powered by fierce and unmistakable moral indignation, and successful so far as his readers approved of it. It was a piece of ethics. The wider program of attacking or justifying morality as such remains obscure.

It was therefore no purpose of my book to provide defence against this hypothetical general attack. I am interested, as most serious moral philosophers have been, in explanation rather than in instant justification, in understanding rather than in proof. For ethics as for any other area of thought, we need to *understand* our basic concepts, to resolve conflicts among them, and to relate them systematically to those used in relevant neighbouring fields. That is the sense in which people speak of the 'foundations' of mathematics. Nobody supposes these to be a set of non-mathematical arguments, guaranteeing mathematical truths from outside. In the case of ethics, however, people do for some reason tend to expect this, and even when they give their attention to the concepts, have a remarkable tendency to insist that they shall be simple. Hume's question, 'whether the foundation of morals lies in reason or in feeling?' has been only one of a whole series of grossly over-simple dilemmas, which it is the point of my book to defuse. I draw attention to the complexity of the relevant facts, to the unreality of various simple rulings, and particularly of those, fashionable in the first half of this century, which isolated moral thinking from the natural facts. During this epoch, a variety of reasons were given for tabooing many useful, indeed

essential, concepts such as 'natural' which refer to facts, but are necessary to explain our value judgments. I do not think I can now trespass on your space with a full answer to Professor Sutherland's worries about my treatment of these. But perhaps it will help if I emphasize that I do regard them as explanatory, and not as an underhand short cut to justifying local prejudice.

Yours faithfully,

MARY MIDGLEY
University of Newcastle upon Tyne, UK

East Europe: valuable information

SIR, The need by science of 'extensive, systematic and friendly international ties' noted by your correspondent, Lee Lorch, (13 September, p 98) is one which I am confident is recognized by *Nature* and by the overwhelming majority of its readers. Having worked at various times in research laboratories in half a dozen countries, including the USSR, I have personally had the good fortune to benefit directly from existing ties and have, I hope, made a modest contribution to furthering them.

Lee Lorch accuses *Nature* of allowing itself to be used by propagandists "anxious to whip up an hysterical ill-will against East European science", an accusation so far-fetched that it would not merit refutation if it were not coupled with a scornful and vicious personal attack on your regular contributor, Vera Rich.

Her articles are a valuable source of information about science and technology in Comecon countries. Of course it is incomplete, of course it is biased; Ms Rich writes rather more often on environmental matters than on high energy physics, and as far as I know has never written about Polish research in general relativity. But omission and bias are not the same thing as prejudice, still less contempt and propaganda.

To be sure there have been occasions, sad to say not infrequent, when she has reported on the action of the authorities against individual scientists, their dismissal from employment, arrest, or worse. The cases which she has reported have been in the USSR and in East Europe — but that is the region of her specialist interest and knowledge and others in your journal have balanced the record of injustice with reports from elsewhere.

The achievements of Soviet science are beyond question; the distinction of her scientists and engineers and their contributions to the advancement of knowledge command the admiration and respect of the world community of science. The mutual benefits that flow from international cooperation, the free exchange of ideas and the unrestricted travel of scientists themselves are widely recognized. Your journal indeed has a responsibility, the greater because of its eminence, to report on developments in science in East Europe as elsewhere. But it has a responsibility too to report with the candour and good faith of a true friend of international cooperation the less favourable aspect of the policies of those countries insofar as they affect science and scientists. To pretend otherwise is no way to encourage the ties of friendship: it is to acquiesce in injustice.

Yours faithfully,

JOHN M CHARAP
Queen Mary College, University of London

'MHC' nomenclature

SIR, — Rolf Zinkernagel is too modest (14 June, page 618). The revolution which he and David Katz started has changed our thinking about the 'MHC' (major histocompatibility complex) to an extent which makes nonsense of the conventional nomenclature used in the otherwise excellent book which he reviews. His thesis is now generally accepted that MHC-coded molecules (other than complement components) evolved as signals for T cell subsets, which enable the immune system to make an appropriate choice between these subsets when combatting varied forms of infection. If one takes this point of view, present nomenclature is quite misleading. The very term, 'major histocompatibility complex' place the emphasis on transplantation, not infection. It perpetuates the historical accident which led to its discovery, but misleads as to its normal function.

Terms such as cytotoxicity-defined or lymphocyte-defined are open to the same criticism. The term 'I region' with the associated terms Ia-molecules, I-A, etc. implies falsely that H-2K and D molecules are not immunoregulatory. In fact, they are so, and in a way which appears exactly to parallel the immunoregulatory functions of Ia-molecules, except that they control cytotoxic T cells rather than helper T cells. Furthermore, gene substitutions at K and D regulate immune responsiveness of cytotoxic T cells just as substitutions in the I region regulate the responsiveness of helper cells. The whole complex, again with the exception of the complement components, seems to be equally immunoregulatory. Indeed, one really ludicrous usage is creeping in: to refer to the I-region genes as *the* IR genes. No doubt, many if not most gene substitutions perturb the immune system, just as most known substitutions perturb the nervous system: it's just that behavioural effects are more obvious.

We hesitate to suggest alterations, chiefly because it annoys others so much when one does so. T cell-activating complex? Cytotoxic cell activating molecules versus helper cell activating molecules? There are plenty of problems still: the aberrant presence of Ia-molecules in glandular epithelia and T cell products, the odd behaviour of I-J molecules, questions about restriction elements on B cells, and so on. What is certain is that so long as the present nomenclature is used we shall have to go on explaining to newcomers that the only way to understand the MHC — and hence what the majority of cellular immunologists these days are up to — is to forget about what the terms seem to mean.

Yours faithfully,

J. MICHAEL CECKA, ALEXANDER KAMB,
URSULA KEES, N. AVRIEN MITCHISON
University College, London, UK.

Radio and the 'experts'

SIR, "A pity" — you say (23 August, page 619) — that more qualified people fail to participate in phone-in and open-line radio programmes.

The reason is simple: most of us are at work, so don't hear the programme anyway; those not at work presumably would not wish to advertise the fact!

Yours faithfully

I.P.C. GAUT
Optrex Ltd, Hampshire, UK.

news and views

Vanadate — a new tool for biologists

from T. J. B. Simons

RESEARCH on the biological actions of vanadate has increased enormously during the past three years. Vanadium compounds can be highly toxic to man and animals and yet removal of the minute quantities normally present in the diet leads to deficiency disorders in experimental animals¹. The biological basis for the apparent requirement for vanadium is still unknown but currently its effects on certain enzymes — notably the ATPases — are being studied intensively.

The stimulus to the present interest in vanadium came when vanadate was identified^{2,3} as the impurity in ATP derived from equine muscle, which inhibited dynein ATPase⁴ and the membrane (Na + K)-ATPase^{5, 7}. The impurity was present at only 1/3,000 the molar concentration of ATP.

Since then studies on both the physiological and the biochemical effects of vanadate have revealed a wide range of activity. Particularly interesting is its apparent selective inhibition on some ATPases, which has led to its use as a selective probe. It also inhibits several other enzymes (acid phosphatase⁸, alkaline phosphatase⁹, phosphofructokinase¹⁰ and adenylate kinase¹¹; on the other hand it stimulates adenyl cyclase¹². It can replace phosphate as a substrate for glyceraldehyde 3-phosphate-dehydrogenase. This leads to the formation of an unstable analogue of 1,3-diphosphoglycerate, similar to that produced by arsenate¹¹. Its physiological effects include diuresis, natriuresis¹³, increasing the force of contraction of the heart¹⁴ and vasoconstriction¹⁵.

Two forms of vanadium in particular have been studied and have been found to have different effects. The first, vanadate, exists mainly as H_2VO_4^- anions in dilute solutions at neutral pH, but it polymerises at concentrations above 10^{-4} M (ref. 16). Monomeric vanadate is structurally analogous to phosphate and this could account for its inhibitory effects on enzymes concerned in phosphate transfer or release reactions. Like phosphate, vanadate enters human red blood cells by the anion transport mechanism¹⁷. Inside

cells however, vanadate often seems to be reduced to vanadyl ions (VO^{2+}) which bind to proteins¹⁷⁻¹⁹. Vanadyl ions have different effects from vanadate: they are powerful inhibitors of alkaline phosphatase, but seem not to affect the (Na + K)-ATPase^{18, 20, 21}. One complicating factor in experiments is that vanadyl solutions are readily oxidised to vanadate by the atmosphere.

Inhibition of (Na + K)-ATPase

Cantley and Josephson have done much to characterise the inhibition of (Na + K)-ATPase by vanadate (ref. 22 and see refs therein). (Na + K)-ATPase is embedded in cell membranes and couples ion movements — Na efflux and K influx — to the hydrolysis of ATP. This is brought about through a phosphoenzyme intermediate, whose formation and breakdown are promoted by Na and K ions, respectively. The enzyme is dimeric, and shows both high and low affinities for ATP-binding and ATP hydrolysis²³. The two halves are thought to be coupled in an anti-parallel fashion, with phosphorylation occurring on one subunit at about the same time as dephosphorylation occurs on the other²⁴. Vanadate is a reversible inhibitor. It binds most strongly in the presence of high concentrations of Mg and K ions, when 40 nM vanadate gives 50% inhibition². Detailed binding and competition studies show that it binds to two sites — a high and a low affinity site, and that vanadate at its high-affinity site competes with ATP at its low-affinity site²². Phosphate is a poor product inhibitor, and competes for the high-affinity vanadate site. This raises the possibility that the phosphate release site may be the same as the low-affinity ATP-binding site. It could imply that the ATP-binding sites go through a cycle of high and low affinities towards ATP, corresponding to phosphorylation and dephosphorylation, respectively. Supporting evidence is tenuous as yet: micromolar concentrations of vanadate only inhibit ion transport from the inner surface of the membrane^{17, 26}, where ATP acts.

Another interesting point concerns the promotion of vanadate binding by external K ions. This requires much higher concentrations of K than are needed to

stimulate the (Na + K)-ATPase, suggesting that in this case K acts at sites different from those which normally activate the enzyme²⁶⁻²⁸. When K is absent, ATP is still hydrolysed and Na efflux continues, but at slower rates. The enzyme now shows only high affinity sites for ATP²³, and vanadate inhibits the ATPase with relatively low affinity²². Internal vanadate has no effect on the Na efflux from squid axons in K-free sea water²⁶, in agreement with this observation.

Inhibition of dynein and other ATPases

Dynein is an energy-transducing protein responsible for the mobility of cilia and flagella. It can be extracted from them and shows a Mg-ATPase activity that is 50% inhibited by 0.1 to 1 μM vanadate^{29, 30}. Early experiments showed that vanadate does not inhibit myosin or actomyosin ATPases, except at much higher concentrations^{29, 31, 32} and so can be used as a selective inhibitor to investigate cell motility. A difficulty with this kind of investigation is that the cell membrane is relatively impermeable to vanadate. For example, the flagella of sea urchin sperm are unaffected by up to 10 μM vanadate, but their beating is stopped by as little as 4 μM after the plasma membrane is treated with detergent³⁰. Perhaps the most interesting observation has been made by Cande and Wolniak³², who found that 10 to 100 μM vanadate inhibits chromosome movement and spindle elongation at anaphase in lysed mammalian tissue culture cells, which suggests a role for dynein in the spindle mechanism. More recently, Goodno has found that vanadate really is a powerful inhibitor of myosin, forming a ternary complex with ADP, but the onset of inhibition is so slow that it had previously been overlooked³³.

The sarcoplasmic reticulum (SR), and many cell membranes, have a Ca-ATPase, which couples Ca ion transport (out of cells or into the SR) to ATP hydrolysis. The SR enzyme has many structural similarities to (Na + K)-ATPase³⁴, so it is rather surprising that low concentrations of vanadate have no effect on it³¹, although they do inhibit the red cell Ca-ATPase³⁵. This point needs further investigation. The active transport of Ca out of squid axons seems to be partly dependent on external

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Na, and partly on internal ATP. Vanadate selectively inhibits ATP-dependent Ca extrusion³⁶, which suggests that it is brought about by a Ca-ATPase similar to that in the red cell membrane. Selective inhibition by vanadate might therefore be used to investigate the mechanism of Ca extrusion from heart, smooth muscle and other cells.

Physiological effects

There is an extensive literature on the toxic effects of vanadium salts, and some current observations, of effects on the heart or circulation for example, are not really new³⁷. Recent work has shown that it exerts a positive inotropic effect (increases the force of contraction) in ventricular muscle, but a negative inotropic effect on cat and guinea-pig atria^{39, 40}. Since the (Na + K)-ATPase in membrane preparations from both ventricles and atria is inhibited by vanadate, it must act on cell components other than the (Na + K)-ATPase. Action potential duration is lengthened by vanadate in guinea-pig papillary muscle (ventricle), yet shortened in atria³⁹. This correlates well with the inotropic effect. Catecholamines have a

positive inotropic action on the heart lengthening the action potential, and augmenting inward Ca current. This is associated with a stimulation of adenylyl cyclase and a rise in intracellular cyclic AMP⁴¹. Vanadate is known to stimulate adenylyl cyclase^{12, 40}, so this could account for its positive inotropic actions, but not the negative effect in the atria. Obviously, much work remains to be done to understand the actions of vanadate on the heart.

Recently, deSousa and Grosso reported that vanadate blocks the cyclic AMP-induced stimulation of Na and water transport in amphibian epithelia⁴². The first finding, that Na transport is inhibited, can easily be explained by inhibition of (Na + K)-ATPase. The block of vasopressin- or isoprenaline-stimulated passive water permeability is much more interesting. Vasopressin increases the water permeability of many epithelia. This is associated with a rise in intracellular cyclic AMP, and can be mimicked by exogenous

cyclic AMP. Cyclic AMP is thought to affect the phosphorylation of one or more membrane proteins that control permeability⁴³. The effect of vasopressin can be blocked by colchicine, a microtubule-disrupting agent. This observation has led to the suggestion that microtubules are involved in the action of vasopressin⁴³. Dynein is associated with microtubules, and can be inhibited by vanadate. Perhaps it also plays a part in the vasopressin response⁴².

A regulatory role for vanadate?

Several authors have suggested that vanadate may have a regulatory role in mammalian tissues^{2, 18, 22, 30}. Small concentrations of vanadium are present, and these would be sufficient to affect the (Na + K)-ATPase, if it were present as vanadate. Regulation could be provided by controlling the vanadyl/vanadate redox reaction. However, all that can be said at present is that there is no evidence for or against this hypothesis. □

The genesis of a void lattice

from Robert W. Cahn

WHEN a metal crystal is irradiated with neutrons or other more massive projectiles then, within certain temperature limits, it acquires a population of microcavities known as 'voids': these arise as a result of the preferential agglomeration of the crystal vacancies which are formed together with interstitial atoms as a result of irradiation. In some metals, voids have been found to arrange themselves in an orderly three-dimensional array — a 'void lattice'. Properly speaking, this is a superlattice, parallel to but coarser than the underlying crystal lattice. The classic instance is the void lattice in irradiated molybdenum, which mimics the body-centred crystal structure of molybdenum itself: but whereas the interatomic distance in the molybdenum lattice is a fraction of a nanometre, neighbouring voids in the void lattice are separated by a distance of 18–27 nm, according to the temperature at which it is created.

A very extensive and effective research effort has developed during the past decade into the genesis and growth of individual voids in the alloys used to contain nuclear fuels and also into the inhibition of void formation; but the origin of the void 'lattice', which is of purely fundamental interest, has only intermittently attracted attention. This is a pity, because the phenomenon is intriguing.

A short paper has just been published in which the known facts concerning void

lattice formation in molybdenum are assembled and complemented by some further measurements (Liou, Smith, Wilkes & Kulcinski *J. Nucl. Mater.* **83**, 335; 1979). Molybdenum is the metal in which the existence of a void lattice was originally discovered by Evans in 1971. The new feature which Liou and his collaborators have now established is that voids are originally formed (within a temperature range of about 800–1100 °C) always in a random pattern, and a void lattice is formed only later when a much higher radiation dose has been absorbed and individual voids have grown to a sufficient size. Any theory of the formation of the void lattice therefore has to be based upon the progressive ordering of an initially random dispersion of voids, and indeed such a theory must come to grips with the question of why voids have to grow to a certain size before a lattice can form. Notions such as the nucleation of voids on an ordered pattern of dissolved gas atoms or on an ordered array of dislocation loops (such an array has been observed) are not acceptable.

The stability of a void lattice has been satisfactorily explained by Harwell theorists (for example, Stoneham *J. Phys. F* **1**, 778, 1971). Spherical voids would attract each other at all separations in an elastically isotropic medium, and it is only in an anisotropic medium that a pair of voids can rest stably at a particular separation. Stoneham has proved that a b.c.c. or f.c.c. lattice of voids is stable in molybdenum, though the predicted ratio

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of the separation of voids (in the observed b.c.c. void lattice) to the void diameter is smaller than the values actually observed. Stoneham's theory explains why such a lattice is stable once formed, but not how it is established in the first place.

Liou and his collaborators point out that the formation of a void lattice is analogous to the alignment of precipitate plates on different planes to form a preferred, stable array. This is very commonly observed in alloys formed by spinodal decomposition or by ordering, but had not hitherto been properly analysed. The theoretical basis of this phenomenon has now been rigorously treated by Perovic, Purdy and Brown (*Acta Met.* 27, 1075; 1979), who calculate the total elastic energy of a regular array of plate-shaped precipitates in a matrix, the precipitates lying on three mutually orthogonal planes. They show that such an array, subject to certain conditions (primarily a low interfacial energy and a large, elastically accommodated misfit between matrix and precipitate) can stabilise at a particular value of the separation of adjacent precipitates, so that further coarsening of the array is inhibited. This presumably is what happens in the formation of a void lattice, though in fact it is not yet known whether a fine void lattice forms first and then coarsens until it becomes stable, or whether the initially disordered voids move about in a kind of Brownian motion until the relative positions of a small group of voids happen to be correct to nucleate a lattice at its proper stable lattice parameter. In the case of a precipitate array, each precipitate plate creates an anisotropic strain field around itself, and an isotropic medium is assumed, for simplicity; whereas in the case of voids, it is only the anisotropy of the medium which allows a void lattice to form at all.

Electron microscopy has shown that an array of precipitates can coarsen progressively by simultaneous dissolution of some portions of a precipitate plate and growth of other portions (Saunderson, Wilkes & Lorimer *Acta Met.* 26, 1357; 1978), and voids or gas bubbles are known to migrate and merge by a similar mechanism. The growth of voids can be inhibited by small amounts of impurity, which may be fission products such as technetium; the impurity can gather at the void wall and prevent plating out of further vacancies into the void. Because of this, voids may even shrink in the later stages of irradiation (Evans *J. Nucl. Mater.* in the press). In such instances, the eventual shrinkage of the void population is in competition with the formation of a void lattice (which requires a minimum size of void to be attained). What is really required now is simultaneous ion-bombardment and electronmicroscopic observation of the same samples so that the stages of formation of a void lattice can be observed while they actually happen.

Most published photographs of void

lattices show them to be distinctly defective (for instance, the photograph on page 338 of Liou's recent paper); the periodicity has a good deal of scatter. It would be interesting to know whether a void lattice, once formed, gradually loses such regularity as it possesses if it is progressively heated; just as a conventional superlattice in, say, Cu_3Au gradually disorders as the alloy is heated. No experiments on annealing of void lattices seem to have been attempted, nor has anyone attempted to improve the regularity of a void lattice by very slow cooling (which is the method used to obtain highly perfect conventional superlattices). There are plainly a number of parallels between void lattices and conventional superlattices which deserve critical examination. □

A spherical structure for allophane

from Ian Smalley

KOJI Wada of Kyushu University has produced and collected evidence which strongly suggests that the clay mineral allophane, long regarded as an 'amorphous' constituent of soils, has a unique spherical structure. Although plates and tubes have already been encountered in clay mineralogy, this seems to be the only occurrence of spheres (*Proc. Int. Clay Conf.*, eds Mortland & Farmer, 537; Elsevier, New York, 1978). Taylor and Wilson (*Clays and Clay Miner.* 27, 261; 1979) describe allophane as consisting of an aluminosilicate core, about 5 nm in diameter, coated to a variable extent with amorphous aluminium hydroxide. The clay surface has hydroxyl and hydronium groups associated with the polymeric hydroxide and the aluminosilicate, and these (by their protonation and deprotonation) are responsible for the pH-dependent particle charge developed in aqueous systems. (The nature and properties of variable-charge soils containing allophane will be discussed at the International Society of Soil Science meeting in New Zealand in 1981.)

Allophane usually coexists with imogolite, but the two can be distinguished easily by their shapes, spherical allophane contrasting with tubular imogolite. In 1971 Kitagawa first discovered by high resolution electron microscopy that allophane consists of spherical, hollow unit particles with an average diameter of 5.5 nm. Hemmi and Wada (*Am. Miner.* 61, 379; 1976) observed that similar particles occurred in sixteen allophanic clays, irrespective of their ages and lithological

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compositions, or the origins of the volcanic ashes and pumices from which the clays were separated. They estimated the external diameter of the particles to range from 3.5 to 5.0 nm and the thickness of the wall to be 1.0 nm or less. The thickness of the wall is broadly consistent with that of a defect kaolin structure.

Wada and Wada (*Clay Miner.* 12, 289; 1977) determined the density of allophane by float-sink tests in CH_3OH -, CH_3COCH_3 -, C_6H_6 - and CCl_4 - $\text{C}_2\text{H}_2\text{Br}_4$ mixtures as 1.84, 1.94-1.98, 2.35 and 2.35-2.39 g cm^{-3} respectively. Either water molecules only fill the voids of the spherule wall, or all liquid molecules (including $\text{C}_2\text{H}_2\text{Br}_4$) occupy these spaces, the first possibility applying if the wall contains small pores and the second if it contains large pores. It is reasonable to assume that 'defects' in the silicate structure produce such pores or openings in the wall, and the passage of water molecules through these has been confirmed by the ease with which OH ions can be replaced by their deuterium-containing equivalent (OD^-) in allophane.

Wada concludes that the structural formulae for allophanes are in general consistent with the available electron optical and density evidence. The modification of the kaolin layer caused by the size and shape of the unit particles, and by the occurrence of defects, may account for several features of allophanes, especially the absence of both basal and two-dimensional X-ray reflections, the broadness and poor resolution of Si-O-(Al) absorption bands in the 1100-900 cm^{-1} region of the infrared spectra, and the remarkably continuous loss of water with increasing temperature due to dehydration and dehydroxylation. The observed electric charge and acid characteristics of allophanes are also broadly consistent with what is predicted from the structural formula. □

Soil biology and land use

from Clive A. Edwards

A RECENT symposium* addressed the often neglected subject of the impact of human activities on the soil — the resource on which all plant and animal life depends. Although man's use of the land can often have damaging effects on the soil, work presented at the symposium showed that with care, such effects can be minimised, and careful manipulation of some of the multitude of soil organisms can benefit man by increasing soil fertility, and can help to reclaim polluted land and dispose of an increasing volume of human and animal waste.

There has been much discussion on how

pesticides, used in agriculture and in aerial spraying programmes, affect soil ecosystems and a day was devoted to assessing how pesticides affect soil communities and in particular their effects on earthworm and arthropod populations. Work at Rothamsted Experimental Station in England has shown that relatively few pesticides are toxic to earthworms; those that do kill these animals which are important in maintaining soil fertility, include the insecticides chlordane, phorate, parathion and carbofuran, the nematocides D-D, methyl bromide, metham sodium and chloropicrin, and the carbendazim-based fungicides. Work by A.B. Broadbent and A.D. Tomlin, (Canada Agriculture, London, Ontario) and others has shown that pesticides do not have a drastic overall effect on soil arthropod communities but that rather they tend to upset the precarious balance in soil ecosystems, sometimes leading to spectacular upsurges in numbers of those organisms unaffected by a pesticide when their predators were killed; and even, in some instances, new pest problems.

Keen interest was attracted by the work of Roy Hartenstein and his colleagues (College of Environmental Science and Forestry, Syracuse) on the use of earthworms to process human sewage. As restrictions on the dumping of sewage at sea are implemented so waste disposal problems are becoming more serious. The Syracuse workers have found that certain species of earthworms, particularly *Eisenia foetida*, can break down raw sewage into a soil-like material, rich in nutrients and suitable for addition to agricultural land. This process takes about 2 weeks and the commercial development of such a process on a large-scale basis is being investigated and seems likely to succeed. The earthworms produced may be useful as a protein supplement in animal feed. One obstacle to such a process is the rather large concentrations of heavy metals such as lead and cadmium that are often present in sewage, and which would be undesirable in agricultural land from which they might move into edible crops. One group of workers reported investigations into the uptake of heavy metals into earthworm tissues and methods of neutralising the effects of heavy metals on and uptake into crops grown on sewage-amended soil (C. Andersen, Royal Veterinary and Agricultural University, Copenhagen, Denmark).

A third subject that attracted interest was the effects of different kinds of animal wastes added to agricultural land on soil organisms. Workers at University College, Dublin (J.P. Curry & D.C.F. Cotton) have shown that the initial effects of slurries was to decrease earthworm

populations in soil to which such wastes had been added drastically but within 7 months populations had recovered and greatly exceeded those in untreated soil. The overall conclusion was that the addition of animal waste to soil tends to favour populations of many soil invertebrates, particularly earthworms, so there seem to be few ecological objections to such a practice.

Surface mining is becoming even more widespread and there is keen interest in the reclamation of such land. The effects of mining iron ore, coal and bauxite on populations of soil organisms were

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Neurotoxins in Nice

from R. C. Hider

MANY of the most potent animal toxins known are specifically directed against critical membrane proteins of the neuromuscular system. As a result of their specificity and high affinities, biochemists and physiologists have been using many such molecules to great advantage over the past decade. This point was clearly demonstrated at the recent successful EMBO workshop on neurotoxins held in Nice*, where a largely European audience heard presentations on the acetylcholine receptor, the sodium channel and presynaptic membranes.

Postsynaptic toxins

Cobra venom possesses extremely powerful toxins which cause the paralysis of vertebrate skeletal muscle. These small proteins (molecular weight 6,000-7,000) achieve this by binding tightly to the acetylcholine receptor (AChR) which is concentrated at neuromuscular junctions. A toxin-bound receptor is no longer responsive to the natural agonist acetylcholine. The detailed structure of the acetylcholine receptor has been a controversial subject for some years. At the meeting J.P. Changeux (Pasteur Institute) presented compelling evidence that AChR is a hexamer of six identical subunits of molecular weight 40,000. Preparations of this protein have been successfully incorporated into vesicles and judging from the rapid binding kinetics of a fluorescent agonist, the reconstituted AChR is in a similar state to that found in native membranes. Agonists increase the permeability of these vesicles towards Na ions, the increase being specifically inhibited by α -bungarotoxin (a snake neurotoxin) (Changeux, Heidmann, Popot & Sobel *FEBS Lett.* 105, 181; 1979). A similar mechanism has been established for

discussed. The general impression was that most mining programmes tend to decrease both species diversity and populations of many soil organisms.

The possibilities of restoring these and other highly disturbed sites to normal biological activity are being investigated by D. Parkinson *et al.* (University of Calgary, Alberta, Canada) by adding different types of surface amendments. They found sewage sludge to be the most effective amendment in terms of plant production and microbial development.

It seems clear from the topics discussed at the symposium that the potential for profitable manipulation of soil organisms to minimise the adverse effects of human activities on soil is considerable and merits much further attention. □

native AChR *in situ* by electrophysiological experiments. A problem with reconstitution experiments of this type, as pointed out by Changeux, is that the presence of trace quantities of other proteins could be associated with the ion permeability changes. A report from M. Raftery (California Institute of Technology, Pasadena) could have a real bearing on this point because he proposed, on the basis of a large body of evidence, that the receptor is composed of four different proteins (Vandlen, Wu, Eisenach & Raftery, *Biochemistry*, 18, 1845; 1979). Furthermore he demonstrated that snake neurotoxins bind to two of these components (Raftery *et al. Adv. Cytopharmacol.* 3, 27; 1979). Both Changeux and Raftery use postsynaptic membranes of the electric organ of *Torpedo* in which AChR constitutes up to 40% of the protein. Significantly, Barnard when isolating AChR from mammalian muscle also finds a single protein of approximately 40,000 molecular weight (Shorr, Dolly & Barnard *Nature* 274, 283; 1978). Barnard also considers that the ligand recognition unit is possibly an oligomer of six identical units.

If this relatively simple concept proves to be correct then AChR will soon be subjected to classical enzymological studies. Indeed, Changeux presented the N-terminal sequence of the 40K protein (Devillers-Thiery *et al. FEBS Lett.* 104, 99; 1979) and many advances have been made on the interaction between the snake neurotoxins and this receptor (Maelicke *et al. J. biol. Chem.* 252, 4811; 1977). The X-ray structure of two such toxins are now established, erabutoxin-b (Kimball *et al.*

*The VII International Congress of Soil Biology was held on 30 July-4 August at the State University of New York, Syracuse. The proceedings will be published with financial assistance from the US Environmental Protection Agency.

*EMBO workshop on the Structure and Function of Neurotoxins (24-26 July, 1979) organised by Michelle Lazdunski, Centre de Biochimie, Nice.

Biochem. biophys. Res. Comm. **88**, 950; 1979) a-cobratoxin (Walkinshaw *et al. Proc. 6th Int. Symp. on Animal, Plant and Microbiol. Toxins* in the press) and suggestions that regions on these molecules mimic agonists (Low, *Handbook exp. Pharmacol.* **52**, 213; 1979) and curare-like antagonists (Hider, *Adv. Cytopharmac.* **3**, 147; 1979) were presented at the workshop.

As a result of the specificity of these toxins for AChR, they are used for the isolation of the receptor by affinity chromatography and for monitoring AChR levels and turnover rates in muscle and myasthenic patients (Dolly, *Int. Rev. Biochemistry* **26**, 257; 1979).

The sodium channel

Until recently knowledge of the sodium channel has been largely based on electrophysiological data; however, with the discovery of a number of specific toxins this membrane-bound protein has now become susceptible to biochemical study. T. Narahashi (Northwestern University, Chicago) indicated that this large molecular complex appears to be more complicated than AChR, a fact possibly related to its ability to sense membrane potential. Three major toxin-binding sites are being used to characterise the sodium channel — the ionophore site that binds tetrodotoxin (from puffer fish), together with the two peripheral sites, one which opens sodium channels when occupied by the lipophilic toxins, veratridine (a plant alkaloid) and batrachotoxin (from the Colombian frog), and one which blocks sodium inactivation when occupied by polypeptide toxins isolated from sea anemone and scorpion species. An important limitation to the use of lipophilic toxins emerged when W. Ulbricht (Physiologisches Institute, Kiel) warned that the non-specific detergent properties of veratridine begin to predominate above 10^{-4} M.

W.A. Catterall (University of Washington, Seattle) described his two-state model of the channel (*Adv. Cytopharmac.* **3**, 305; 1979) and on the basis of toxin-binding studies suggested a molecular interpretation of the classical Hodgkin-Huxley model of electrical excitation of squid giant axon. Hodgkin and Huxley showed that the activation of sodium channels depends on the value of two voltage-dependant variables (m and H) according to the expression m^3h and Catterall has demonstrated that the ratio of tetrodotoxin and scorpion toxin sites is approximately 3:1 (Catterall & Morrow *Proc. natn. Acad. Sci. U.S.A.* **75**, 218; 1978). This could account for the original m^3h empirical finding. Lazdunski presented recent work on the development of highly radiolabelled derivatives and photoaffinity labels of tetrodotoxin and

sea anemone toxin (Lazdunski *et al. Adv. Cytopharmac.* **3**, 353; 1979). It is likely that these compounds will have an important part to play in the elucidation of the sodium channel structure. Indeed the sea anemone toxins may well prove to be suitable for affinity chromatography of the sodium channel. J.C. Fontecilla (University of Alabama) announced the 3-D backbone structure of a scorpion neurotoxin (*Centruroides sculpturatus*), the first to be determined by X-ray crystallography. This important finding, together with the controversy concerning voltage-dependent binding make it likely that the polypeptide toxins will be a focus of attention for the next few years.

Surprisingly, no toxins have yet been found with high affinity for potassium or calcium channels.

Neurotoxic phospholipases

The third major topic of the meeting centred on snake venom phospholipases, and it was fitting that H. Fraenkel-Conrat (University of California, Berkeley) should present a paper as he was the first biochemist to purify a snake toxin (*Berichte* **71**, 1076; 1938). This protein, crotoxin, was isolated from the South American rattlesnake and was found to be a phospholipase. His original isolation method is still used today.

Many snake venom phospholipases are extremely potent toxins with a high affinity for neuromuscular junction membranes. P. Strong (University College, London) described the biphasic behaviour of β -bungarotoxin (from the many banded krait) on presynaptic membranes. After an initial binding response which results in a decline of acetylcholine release, a secondary response expressed by an elevated level of release occurs over a 2-hour period. This latter response finally results in the irreversible inhibition of transmitter release. The initial binding response is independent of phospholipase activity, in contrast to the second effect which is completely dependent on such activity (Kelly, von Wedel & Strong *Adv. Cytopharmac.* **3**, 77; 1979). A plausible explanation for this phenomenon is that the toxin initially binds selectively to the plasma membrane and subsequently the phospholipase-active portion of the toxin promotes hydrolysis of the membrane phospholipids. The nature of this specific binding site is not known, although B. Howard (University of California Medical School, Los Angeles) presented evidence to suggest that it would be the Ca translocating system.

One confusing aspect of neurotoxic phospholipases is that many have more than one protein subunit. They all seem to possess a phospholipase unit homologous to porcine pancreatic phospholipase, and this catalytic portion is connected by means of disulphide bonds or electrostatic/hydrophobic interactions to a second nonenzymatic unit. C. Bon

(Institut Pasteur, Paris) suggested that the nonenzymatic subunit of crotoxin, which possesses a net negative charge, enhances the pharmacological efficiency of the positively charged phospholipase component by preventing its absorption to nonsaturable binding sites, restricting its binding to specific sites (Bon & Jeng *Adv. Cytopharmac.* **3**, 231; 1979). A similar mechanism has been suggested by Hendon and Tu (*Biochim. biophys. Acta* **578**, 243; 1979). However, this is unlikely to be a general mechanism for neurotoxic phospholipases as, for instance, the net charge of the nonenzymatic portion (B-chain) is 5^+ , whereas that of the phospholipase portion (A-chain) is close to zero. An alternative function for the B-chain is implicated for this molecule. Surely it is significant that the B-chain is homologous to the Kunitz-type of proteinase inhibitors (Kondo, Narita & Lee *J. Biochem.* **83**, 101; 1978) and that other such homologues have been isolated from venoms of the Elapidae (Joubert & Strydom *Eur. J. Biochem.* **87**, 191; 1978).

It is becoming clear that many of the membrane proteins in excitable tissue undergo conformational changes which can be explained in terms of simple allosteric models. As membrane bound trypsin molecules render lipid bilayers permeable to ions when exposed to typical trypsin substrates it is not difficult to envisage how receptors of the type described by Barnard and Changeux could have evolved from water-soluble enzymes.

Invertebrate respiratory proteins

from E. J. Wood

Because the first X-ray and sequence studies are now appearing, rapid advances can be expected in the next few years in our knowledge of the structure and function of invertebrate respiratory pigments. These proteins, which include the haemocyanins, the erythrocrucorins and chlorocruorins, and the haemerythrins (see Hendrickson, *TIBS*, May 1977 for a brief review), were discussed at a recent EMBO workshop in Tours*.

Haemocyanins are non-haem copper proteins which exist as giant aggregates dissolved in the blood of molluscs and arthropods. Mollusc haemocyanins possess very large subunits, and in gastropods, for example, 20 such subunits are assembled in a helical fashion to produce a 9 million molecular weight structure with a 'collar' at each end. The subunits are thought to be single polypeptide chains each consisting of eight

*The EMBO workshop 'Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins' was organised by J. & F. Lamy at the University of Tours, France, on 20–24 August.

covalently linked oxygen-binding moieties of molecular weight around 50,000, usually referred to as 'oxygen binding domains', in a string. It has proved difficult to determine the exact molecular weight of this basic subunit by physical methods, and SDS-gel electrophoresis does not seem to give reliable values for apparent molecular weights in this range (molecular weight 300,000). Preliminary studies on the biosynthesis of mollusc haemocyanins seem to show that the product of mRNA translation is indeed very large (E. J. Wood, University of Leeds).

From proteolytic digestion studies information on the order of the different types of domain in the string is emerging. (R. Lontie, Katholieke Universiteit, Groningen). Several of the domains are now available in a reasonably pure state and immunological, as well as spectral criteria are used to distinguish them. It seems likely that they differ functionally which may explain certain heterogeneities in functional properties of these haemocyanins.

Arthropod haemocyanins differ from molluscan ones in a number of respects including electron microscopic appearance and their comparatively small subunit polypeptide chains which contain single oxygen-binding sites. Up to seven types of subunits are needed in the assembly of the native molecule in scorpion and *Limulus*, and J. Lamy (University of Tours) has shown how useful immunological techniques are in identifying and quantitating subunits, as well as for helping in the phylogenetic classification of groups of arthropods. Certain subunits share both immunological and assembly correlates in a number of species, and subunit stoichiometry is becoming better understood. Reassembly studies have shown, for example, that for *Eurypelma* haemocyanin, three different types of subunit are needed for the formation of a 16S hexamer, five different for a 24S dodecamer, and all seven for a stable 37S aggregate or '24-mer' (which is the native form). Sequence studies are already showing some homologies between subunits in haemocyanins from *Eurypelma* (a tarantula) and *Androctonus* (a scorpion).

Complete ignorance of the protein structure has so far prevented a description of the changes when O₂ binds to the active site of haemocyanins. A simple model for allostery in haemocyanins was proposed in which Cu—Cu distances affect the strength of O₂ binding and the rate of O₂ dissociation (C. Bonaventura, Duke University Marine Laboratory). Oxygen binding by haemocyanins from both arthropods and molluscs shows certain common features. The discovery that deoxy and carbonmonoxy-haemocyanins (H. Kuiper, University of Rome) have different fluorescence spectra will allow the CO derivatives to be studied in much

more detail than has hitherto been possible.

The binding of O₂ by haemocyanin, which is often cooperative, can be described, approximately at least, by a two-state model in which allosteric ligands may bind preferentially to the T or the R state. M. Brouwer (Groningen) has studied the binding of O₂ by *Limulus* haemocyanin and concluded that the 'switch-over' in state occurred (mostly) as the fourth O₂ was added. However, binding of the allosteric ligands Cl⁻ or H⁺ can change this. The kinetics of the R-T transition are under investigation and it is suggested that the size of the allosteric unit may be correlated with the rate of the conformational change (M. Brunori, University of Rome).

The relationship between the functional properties of haemocyanins and the haemolymph concentrations of such allosteric ligands (as well as of Ca²⁺ and Mg²⁺ which can control the aggregation state of the molecules) deserves more study than it is receiving at present. The fact that many haemocyanin-containing animals have little or no ability to regulate the ionic composition of their blood may lead to a consequential limitation of O₂-transporting properties, or there may be an important physiological adaptation reflected in the effect of these so-called allosteric effectors on the functional properties of haemocyanin (C. Mangum, Williamsburg).

Several groups are working on the big haemoglobins (or erythrocrurins) of annelids and on the rather similar chlorocruorins. Although the molecules occur in haemolymph as enormous aggregates (molecular weights of up to 4 × 10⁶) they have much smaller subunit polypeptide chains than mollusc haemocyanins. However, general rules about the number of subunits and their sizes have yet to emerge, and it has been difficult to agree on the minimal molecular weight per haem based on chemical determinations. One subunit polypeptide chain from the earthworm, *Lumbricus*, haemoglobin has now been sequenced (Riggs *Fed. Proc.* 38, 343; 1979) and has 157 residues corresponding to a molecular weight of 17,500. The sequence shows definite homologies with the β-chain of human haemoglobin. Uncertainties about size and shape of subunits have so far prevented formulation of a definitive model for the native molecular aggregate.

Although the 'annelid type' of haemoglobin is common, even being found in a deep-sea tube-dwelling worm from a hydrothermal vent, as reported by N. Terwilliger (Charleston, Oregon), many other types are known. These range from the monomeric (and sometimes dimeric) chironomid haemoglobins, for which there is much sequence information and which are like myoglobin (T. Kleinschmidt, University of Munich), through what

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might be called medium-sized haemoglobins from *Artemia* (F. De Voeght, Wilrijk, Belgium), branchiopods (E. Ilan, Tel-Aviv) and planorbid snails, to those even bigger than the annelid type such as that from a clam, *Cardita*, reported by R. Terwilliger to have a molecular weight of 8 × 10⁶. In contrast to the annelid type of haemoglobin many of these seem to have very large (presumably multidomain) polypeptide chains.

The third group of invertebrate respiratory pigments comprises the nonhaem iron proteins, the haemerythrins. Very much more is known about these proteins which have only a limited biological occurrence, than about the haemocyanins or invertebrate haemoglobins although admittedly they have a much simpler structure. The coelomic forms (tri, tetra and octomers) have a polypeptide chain of 113 amino acids while the monomeric muscle myohaemerythrin has 118, but there are nevertheless considerable homologies. The coelomic form has four major helical regions (A—D): myohaemerythrin differs in having five extra residues in the C-D corner (W. Hendrickson, Washington). It seems that the polymeric forms possess different subunits, but the differences are minor. The iron site is prominent and resonance Raman studies (D. Shriver, Evanston) have given valuable clues to the mode of oxygen binding. □

100 years ago TAILS

The tadpole is at first a singular object. It consists of a head and body indistinguishably united in one rounded all, from behind which a long and slender tail projects. The creature may be said to be indeed at first "all head and tail."



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review article

Crown gall disease

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Agrobacterium tumefaciens induces tumours in dicotyledonous plants by transferring part of a large bacterial plasmid to the eukaryotic cell. As well as disrupting control of cell division, the transferred DNA determines the synthesis in transformed tissue of novel amino acid compounds which serve as specific substrates for the bacterium. It may prove possible to exploit such DNA transfer for the genetic engineering of higher plants.

CROWN GALL is a plant tumour caused by *Agrobacterium tumefaciens*. It was first described by Aristotle, first assigned a bacterial aetiology by Smith and Townsend¹, and first proved to be a true oncogenic transformation by White and Braun², who showed that crown gall tissue cultured in the absence of the bacterium retained its tumorous properties. The disease occurs in a remarkably wide variety of higher plants³ but can only be induced in freshly wounded tissue⁴. Oncogenic strains of the bacterium contain a large plasmid, part of which is transferred to the plant cell during oncogenic transformation and maintained there indefinitely⁵; to this extent crown gall resembles the virally induced animal tumours. Transformed tissue is distinguishable by its ability to form an overgrowth when grafted onto a healthy plant, its capacity for growth in tissue culture without added plant hormones⁶, and its synthesis of 'opines', unusual derivatives of basic amino acids not found in normal tissue⁷⁻¹⁰. The study of crown gall may help the understanding of carcinogenesis and should be important in elucidating the control of gene expression in higher plants. The possibility of using *Agrobacterium* plasmids as vehicles for genetic engineering in higher plants is also of interest.

Understanding the disease at the molecular level only became possible recently with the discovery that all oncogenic strains of *Agrobacterium* contained large plasmids of about 150–230 kilobases¹¹, which proved to be essential for tumorigenesis. Hamilton and Fall¹² had shown that one bacterial strain became non-tumorigenic when cultured at 37 °C, and this was shown to be invariably associated with plasmid loss¹³⁻¹⁶. When other *Agrobacterium* plasmids were introduced into the cured strain by conjugation or transformation it recovered oncogenicity, confirming the involvement of the plasmid in oncogenesis¹⁶⁻²¹. Not all large *Agrobacterium* plasmids confer the ability to induce tumours; those that do are designated Ti (tumour-inducing) plasmids. None is smaller than 140 kilobases. So far relatively few genetic markers have been ascribed to them.

The catabolic function of Ti plasmids

The metabolism of opines is a central feature of crown gall disease. Axenic crown gall tissue synthesises opines which can serve as a carbon or nitrogen source for oncogenic agrobacteria. Morel and coworkers noted that the nature of the opine produced is determined not by the host plant but by the bacterium, and is invariably the one catabolised by the inducing strain^{22,23}. They interpreted this correlation as evidence for

DNA transfer from the bacterium to the plant cell, and suggested that the enzyme catabolising a given opine in the bacterium was identical with that producing it in tumour tissue. In fact two different enzymes are involved, distinguishable both genetically and biochemically^{8,24-28}. The enzyme responsible for bacterial catabolism is referred to as an opine oxidase, while that in the plant is termed a synthase. Both are probably plasmid-encoded, although this has not yet been demonstrated unequivocally. With a single exception, the Ti plasmids isolated so far code for the synthesis and breakdown of either octopine or nopaline but not both. The substrate specificity of synthases and oxidases is, however, not exact and both octopine and nopaline tumours contain a characteristic family of opines, comprising analogues of octopine or nopaline (see Fig. 1), which can also support the growth of the inducing strain of *Agrobacterium*²⁹⁻³⁴. Most octopine tumours also contain massive amounts of the recently discovered opine agropine, which is probably the product of a separate synthase, as it is absent from some tumours that do contain octopine (J. L. Firmin, personal communication), and stereochemically is quite unlike octopine³⁵. The finding that the plasmid pTi542 codes for agropine metabolism alone bears out this supposition (J. Tempé and J. L. Firmin, personal communications). Three distinct plasmid-encoded permeases exist for nopaline, octopine and agropine^{35,36}. The expression of oxidases and permeases by octopine and nopaline determining plasmids has been shown to be coordinately controlled, both enzymes being inducible by their particular substrate³⁷. In some strains, the catabolism of arginine, a breakdown product of both octopine and nopaline, is a plasmid-borne trait which is also inducible by the appropriate opine³⁸.

Ti plasmids promote bacterial conjugation^{39,40}, and the genes required for their transfer (*tra* functions) are closely associated with opine metabolism, being inducible by the characteristic opine^{41,42}. This makes good teleological sense, as in the presence of its substrate the catabolic plasmid will spread rapidly through a mixed population of oncogenic and non-oncogenic bacteria. The three classes of regulatory mutant that can be isolated are most simply explained by postulating separate operons for plasmid transfer and opine catabolism, controlled by the same repressor⁴³⁻⁴⁵.

Tumorigenesis is almost always accompanied by opine synthesis, but as one can isolate mutant plasmids that give tumours failing to produce any opine, such synthesis cannot be necessary for tumour maintenance^{45,46}. However, there is some evidence that opines enhance tumour growth^{47,48}. Perhaps the

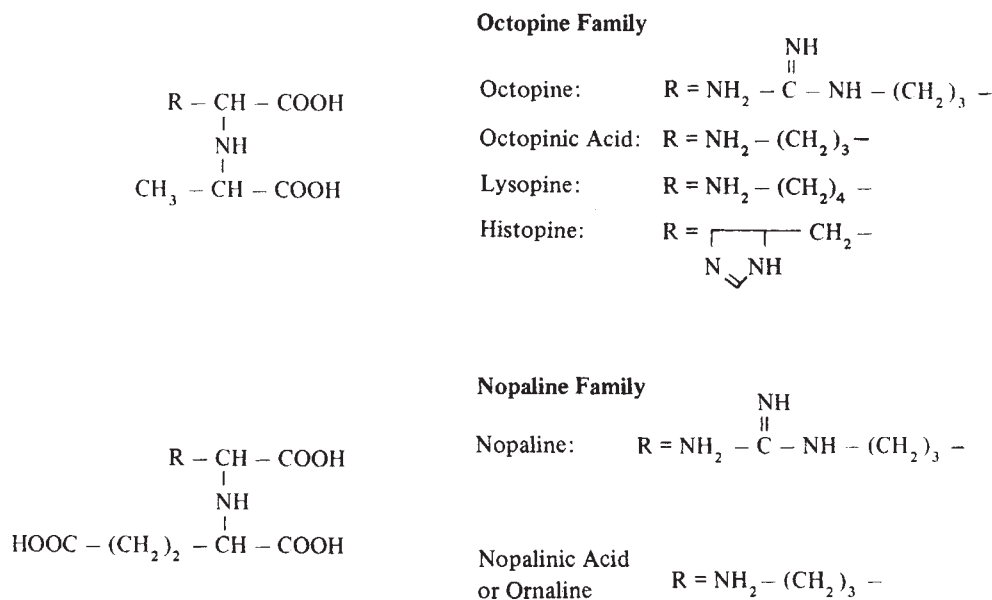


Fig. 1 Structural formulae of opines synthesised by crown gall tumours.

most plausible rationale for the presence of opines in tumour tissue is that they provide a specific ecological niche for the oncogenic bacterium⁴³. As only bacteria capable of using opines as carbon and nitrogen sources can exploit the niche, the plasmid thereby generates selection pressure for itself as well as promoting its own transfer through the population. The logical link between opine production and uncontrolled cell division is that the plasmid need only transform a single cell to procure a relatively abundant supply of its opine substrate. It is quite conceivable that other plasmids are also capable of introducing some of their genetic information into eukaryotic cells but go undetected because they lack the more conspicuous oncogenic phenotype.

The opine strategy of the Ti plasmids can be seen as an example of altruism at the molecular level. Altruism is a property of a gene which reduces the darwinian fitness of the individual while enhancing the reproductive potential of the population to which it belongs. T-DNA in the plant cell cannot ultimately enhance its own chances of survival as it must perish with the somatic tissues of the plant, but its expression increases the chances of replication of T-DNA that remains part of the bacterial replicon.

Models of tumorigenesis

A complete description of the crown gall transformation must explain how plasmid DNA enters the plant cell, how it manages to be replicated in the eukaryotic genome and how it exerts its

characteristic physiological effects. Much of this is now known, but a full description involves some speculation.

The absolute requirement for a wound for tumorigenesis in the whole plant must be accounted for. Wounding might be necessary to expose bacterial attachment sites on the plant cell surface. A lipopolysaccharide element of the bacterial outer membrane is known to adsorb to specific sites on the plant cell wall^{49,50}. An alternative explanation postulates a need for cell division associated with wound healing for successful oncogenesis. This is more consistent with the finding that only if a lesion is inoculated with *Agrobacterium* before the first wave of cell division—up to 2 days after wounding—does oncogenesis proceed as usual^{51,52}.

The formal resemblance of the plasmid transfer phase of oncogenesis to bacterial conjugation may be reflected at molecular level. Whether the entire Ti plasmid enters the plant cell is not known, but both transformation and conjugation are temperature sensitive with critical temperatures around 30 °C, suggesting that one or more of the oncogenic (*onc*) and transfer functions could be identical^{42,53}. However, mapping of the *tra* and *onc* genes in both octopine and nopaline plasmids has not yet revealed any overlap of these functions^{45,46}.

As well as the genes for plasmid transfer, octopine and nopaline Ti plasmids presumably share at least some of the genetic information involved in oncogenesis. The extent and distribution of DNA sequences conserved on all Ti plasmids is thus relevant to the mechanism of transformation. Octopine plasmids are all very similar in their restriction patterns and

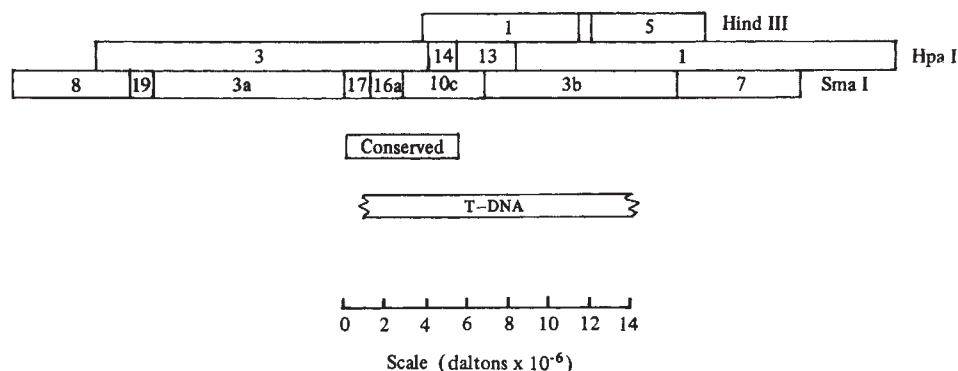


Fig. 2 Section of the physical map of the typical octopine plasmid pTiB6-806 showing fragments detected in tumour tissue (T-DNA) and the DNA which is stringently conserved on all Ti plasmids. Redrawn from Chilton *et al.*⁶¹.

sequence homology^{54,55} but are only 20–60% homologous with various nopaline plasmids⁵⁶, which themselves form a much more heterogeneous group⁵². Thus if one assumes there has been no divergence in the *onc* functions, only 20% of the information content of the Ti plasmids can be involved in tumorigenesis. This would raise the question of why the plasmids are invariably so large—perhaps the *onc* genes have diverged. It is clear that the opine determinants have done so, maybe to exploit host plants containing different major constituents of a particular opine, and divergence of the *onc* genes in response to host biochemistry is entirely consistent with the plasmid-borne variations in host range which have been observed⁵⁷.

Chilton *et al.*⁵ demonstrated the presence of octopine plasmid DNA in a cloned tobacco tumour by using an entire set of plasmid restriction fragments as probes in a series of solution hybridisation experiments. They found ~20 copies per cell of a 6-kilobase segment representing about 3% of the plasmid. Such stretches of plasmid DNA, designated T-DNA, are probably integrated to the plant genome⁴⁶. The mechanism of integration is unknown, but may well differ from that of prokaryotic transposons as stretches of T-DNA derived from a single cell can vary in length⁵⁸.

Experiments with restriction fragments containing T-DNA of an octopine plasmid show that the left hand end of the octopine T-DNA and sequences extending as far as its left-hand boundary are highly conserved on all Ti plasmids^{59,60} (see Fig. 2). Heteroduplexes of this region show 3–5% base mismatching, markedly lower than the 9–12% mismatching observed for DNA sequences conserved elsewhere on the plasmid^{56,60}. Experimental integration of the plasmid RP4 into its area of highly conserved sequences renders a Ti plasmid non-oncogenic⁵⁹. This DNA segment must therefore function in oncogenesis. The greater part of the conserved region is found in all tumour lines that have been examined and is probably sufficient for tumour maintenance^{46,58,61}. A 6-kilobase *SmaI* restriction

enzyme fragment common to the majority of Ti plasmids encompasses about half of this DNA^{60,61} (Fig. 2). The T-DNA to the right of the stringently conserved segment on the octopine plasmid has little or no discernible homology to the corresponding region on the various nopaline plasmids^{59,60}. Restriction mapping of insertion mutants of both octopine and nopaline plasmids indicates that this region either contains the opine synthase gene or at least contains the gene that controls opine synthesis by the host⁴⁶.

There are two possible ways in which T-DNA could exert its physiological effects. Oncogenesis could result simply from the insertion of T-DNA onto host genes crucial to the control of cell division. The presence of T-DNA in variable copy numbers per cell, and the phenomenon of phenotypic reversion (see below) are, however, easier to accommodate in a model involving a specific oncogenic gene product, analogous to the T-antigen of SV40-transformed rodent cells⁶².

At least part of the T-DNA is transcribed in the plant cell. If ³²P-labelled RNA from an octopine-producing tumour is hybridised to Ti plasmid restriction fragments that have been transferred to cellulose nitrate paper by Southern's method⁶³, it binds strongly to *SmaI* fragment 3b, which includes the right-hand boundary of the T-DNA⁶⁴. The labelled RNA is polyadenylated⁶⁵ and must include the transcript of the octopine synthase gene if that gene is plasmid encoded. In the nopaline tumour examined by Yang *et al.*⁵⁸, there appear to be at least two plasmid RNA species present, indicating that T-DNA sequences other than those coding for nopaline synthase are also transcribed. At least one of these RNA species appears to be from the highly conserved T-DNA, and transcripts from this region have also now been detected in octopine tumours^{65,66}. The conserved DNA could encode the putative oncogenic gene product.

Hormone imbalance is strongly implicated in the aetiology of crown gall disease. Cytokinins, which promote cell division, are abundant in crown gall tissue, as are auxins, a class of plant

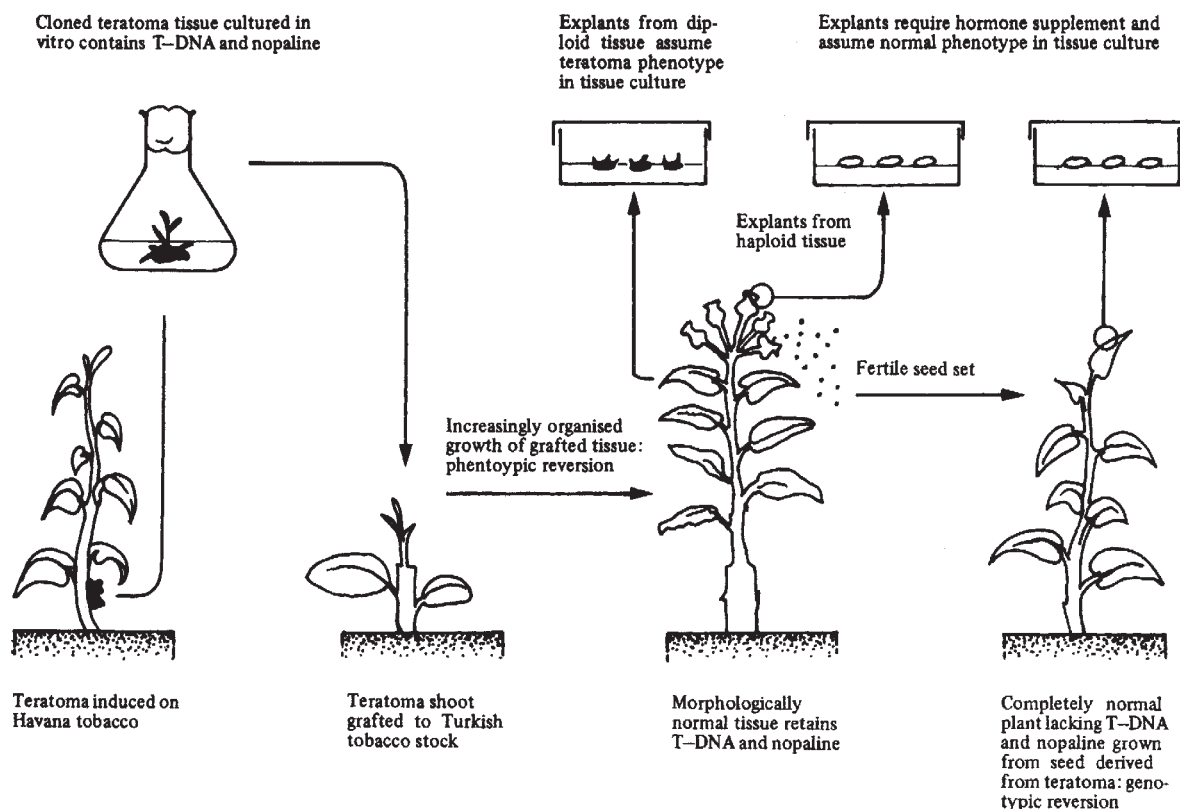


Fig. 3 Reversal of the crown gall transformation.

hormones which stimulate cell enlargement^{67,68}. Untransformed tissue cultured *in vitro* does not contain physiologically effective levels of either hormone⁶. Furthermore, plant tumours caused by Black's wound tumour virus as well as those occurring spontaneously in interspecific hybrids of *Nicotiana* also contain abnormally high levels of these hormones^{69,70}. Such hormone autotrophy might thus be a sufficient condition for plant neoplasia in general.

Reversion

Crown gall tumours vary considerably in their morphology, some producing recognisable stems or roots while others remain a chaotic mixture of cell types. Ti-plasmid-borne genes determine morphology to some extent⁷¹. Nopaline tumours typically organise stem primordia on their surfaces, in which case they are referred to as teratomata. When teratoma shoots are grafted to the apex of a normal plant they are forced into rapid growth and gradually regain normal morphology^{72,73}, a phenomenon termed phenotypic reversion (see Fig. 3). The use of cloned teratoma tissue removes the possibility of selection of untransformed cells, and morphological markers serve to distinguish clearly the scion from the stock. The redifferentiated plant contains T-DNA⁵⁸, synthesises nopaline⁷⁴, and reverts to the tumour phenotype when explanted into tissue culture⁷⁵. This suggests that phenotypic reversion has an epigenetic basis, in which case studying it should be fruitful in understanding control of gene expression in higher plants.

The crown gall transformation may also be reversed at the genotypic level. Redifferentiated teratoma shoots sometimes flower and set viable seed. Plants grown from this seed lack T-DNA⁵⁸, do not synthesise any opine and require a hormone supplement in tissue culture⁷⁵; their recovery is apparently complete. This recovery appears to occur during meiosis, as haploid tissues derived from anther cultures also fail to synthesise nopaline and show the normal hormone requirement in culture. Such complete recovery occurs only very rarely in diploid tissues⁷⁵. Loss of T-DNA at meiosis could occur in one of three ways: (1) haploid progeny carrying T-DNA are not viable (the integration of T-DNA could cause recessive mutations); (2) chromosomes bearing such insertions are specifically excluded from the progeny; or (3) there exists some machinery in the meiotic cell which can excise extraneous material from the genome (controlling elements in maize survive meiosis, however). None of these possibilities can be excluded at present.

Genetic engineering with Ti plasmids

It should be possible to use Ti plasmids as vectors to insert a chosen DNA sequence into the genome of any dicotyledonous plant. The phenotypic reversion described above shows that this need not disrupt normal differentiation, but genotypic reversion may limit the technique to plants that can be propagated vegetatively. The difficulties involved in introducing a bacterial gene into a crop plant and having it expressed appropriately are daunting. For example, simply inserting *nif* (nitrogen fixation) genes into the potato genome would be useless, as to be of value their expression must be subjected to eukaryotic control and some system devised to protect the nitrogenase from oxygen poisoning. More immediate applications of the technique may involve restoring to their original genome plant DNA sequences which have been modified *in vitro*, say to increase the lysine content of a storage protein.

The first step in any such project would be to identify and probably isolate the DNA sequence encoding the desired trait. This must then be inserted into the T-DNA. To introduce foreign DNA sequences into a prokaryotic cloning vehicle it is sufficient simply to cut the vehicle into one or two pieces with a suitable restriction enzyme, then reassemble it with the 'passenger' in place. The Ti plasmids, however, being so large, are extensively cleaved by the available restriction enzymes, and the chances of reassembling a functional whole with the

passenger in the desired site are remote. This difficulty could possibly be obviated by deleting unnecessary segments of the plasmid or by modifying restriction sites where cleavage is not wanted. An alternative would be to use a restriction enzyme such as *HphI* that cleaves at some distance from its recognition site⁷⁶; the 'sticky' ends generated at each cut are then unique, which guarantees accurate reassembly of the plasmid. The passenger could be inserted into an isolated fragment containing T-DNA before the whole is reassembled.

However, it might be possible to avoid manipulating DNA *in vitro* altogether. Schell and his collaborators allowed the transposable element Tn7, which codes for streptomycin resistance, to hop into the T-DNA of a nopaline plasmid. The modified T-DNA was then introduced into the tobacco genome, where it was detected using Southern's⁵³ technique. The chosen genetic trait is unlikely to be a transposable element, of course, but one could attempt to make it so. Some transposons have been shown to be flanked by insertion sequences^{77,78} which may well be sufficient to promote transposition⁷⁹. It should be possible to construct a bacterial plasmid having an insertion sequence at either end of the DNA sequence one wishes to transpose into the T-DNA.

Whether or not it proves possible to exploit the peculiar properties of the Ti plasmids for genetic engineering, work on crown gall disease will continue to make an important contribution to our understanding of the molecular biology of higher plants.

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articles

Crust of oceanic affinity in Iceland

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Evidence for subsidence of at least 2 km during crustal formation and the absence of an increase in minor intrusion density with depth in the lower 2 km are the major results of the field study of a 3-km vertical section of Icelandic crust.

DURING the past six years major efforts have been made to determine the structure, composition and mode of formation of the oceanic crust. Such investigations are, however, severely hampered by the great depth of water in the ocean basins and the difficulty of deep drilling in the basaltic layer of the crust. An alternative approach is to study elevated mid-ocean ridge segments such as Iceland. Although not underlain by typical oceanic crust, Iceland forms a part of the Reykjanes–Kolbeinsey section of the Mid-Atlantic Ridge and contains a record of eruptive processes at a spreading axis. It is also well exposed and easily accessible for study. A detailed investigation of the Icelandic crust should provide important information on the processes of crustal construction, hydrothermal alteration and magmatic evolution of one part of the Mid-Atlantic Ridge.

The first results from a continuously cored, 1,919-m deep borehole drilled in eastern Iceland, forming the lower part of an intensely sampled 3-km section, are reported here. The section is entirely within seismic layer 2. Overall core recovery was 99.7%. Hole diameter was 7.6 cm down to 1,350 m, then 6.0 cm to the bottom.

The structure of eastern Iceland is relatively simple and consists of a thick succession of deeply dissected Neogene flood basalts which dip gently westwards towards and beneath the Neovolcanic zone of North-east Iceland. Walker¹ showed that the lava pile consists of a series of overlapping groups of flows that thin progressively up-dip. Lava dip decreases from 7–10° near sea level to 2–3° near the 1,000-m summit level. The nature of the regional zeolite grade metamorphism suggests that the basalt pile had a finite limit about 1.5 km above sea level where the intensity of dyke injection approached zero and the lavas were horizontal and unzeolitised.

The drill site (lat 65° 01' N, long 14° 21' W) is located near sea level, 8 km east of the centre of the Thingmuli central volcano, at the head of Reydarfjörður in a geologically and geophysically well known area^{2–6}, where crustal dilation by north–south basaltic dykes is 10% (Fig. 1). Final site selection was on the basis of unpublished gravity data (G. Palmason, personal communication) which were used to identify a location within a large area of anomalously high temperature gradient (~80 °C km⁻¹ (ref. 7)) where the regional gravity anomaly was of average value.

Lava stratigraphy

The combined exposed and drill-hole 3-km section consists of subaerial lava flows, with thin intercalated pyroclastic and volcanoclastic sedimentary horizons, all cut by minor intrusions (Fig. 2a, b). In the drilled sequence minor intrusions comprise 40% of the core and cut out large sections of flow stratigraphy.

Six minor faults with displacements up to 50 m are present in the exposed section and similar faults probably cut the drilled succession, although no obvious stratigraphic repetitions were observed. Regional mapping has indicated the absence of major faults in the area².

All thickness values quoted for both the exposed and drilled sequences are vertical thicknesses. The deviation of the hole from the vertical was measured by acid tests at 100-m intervals and was usually much less than the maximum value of 7°.

The observed dip in the cliff section varied from 2° at 1,000 m elevation to 10–14° at sea level. Dips measured in the drill core are scattered but suggest that the observed increase with depth continues until a value of about 20° is reached at about 500 m below sea level, below which little further increase occurs.

Examination of the drill core in the field showed that the flows are predominantly subaerial fine-grained aphyric tholeiitic lavas characterised by reddened rubbly flow tops and massive to weakly flow-banded grey-green interiors, corresponding to the tholeiites and basaltic andesites of Walker². Notable exceptions include a 12-m thick Icelandic flow from 1,083 to 1,095 m (unit 185.5) and a 31-m thick welded ash-flow tuff sequence from 920 to 951 m (units 157.2, 161.1 and 162.1).

* The members of the scientific party of the Iceland Research Drilling Project are listed at the end of the article.

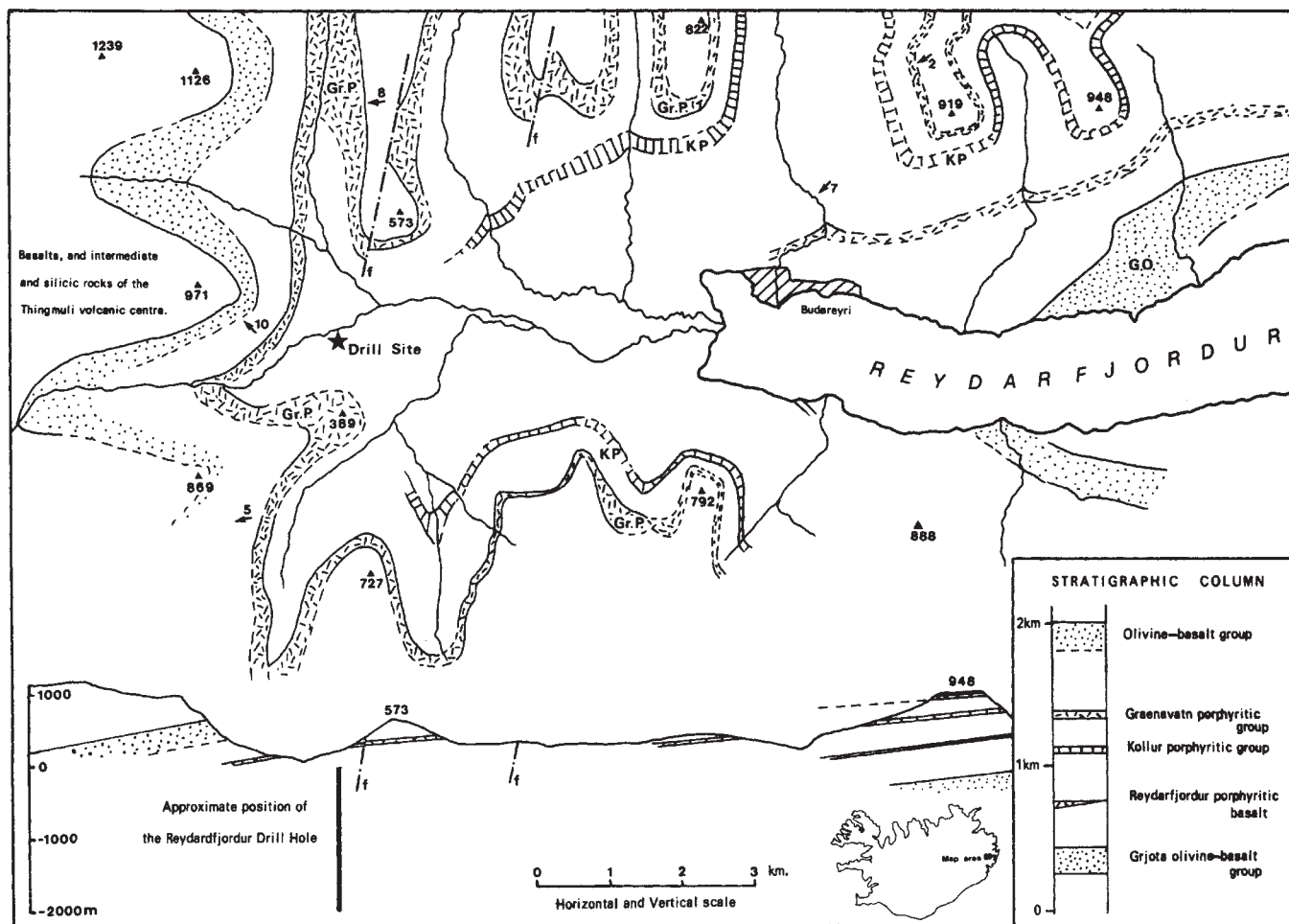


Fig. 1 Map and section showing the geology in the vicinity of the IRDP Reydarfjörður drill site.

It is not possible to correlate the major part of the drill-hole succession with the observed up-dip lava sequences exposed on the north and south sides of Reydarfjörður. This was unexpected in view of the regular nature of the exposed geological structure but several features may explain this situation. First, minor intrusions may cut out some key horizons. Second, an unknown amount of down-dip thickening of the lava units may occur—a simple extrapolation of the rate of thickening observed in the sub-aerial section may not be applicable to the lower part of the section. Third, gradual but large-scale lateral changes in the lava stratigraphy may exist.

Aside from difficulties in making specific correlations between the drill hole and exposed, up-dip sections, a striking general result is the paucity of olivine-bearing basalts in the 3-km crustal section, in contrast with a frequency of occurrence of 23% (ref. 2) in the Reydarfjörður succession as a whole. This paucity of olivine-bearing rocks may relate to the early manifestation of the Thingmuli and Breiddalur centres, which erupted over-saturated basaltic lavas and silicic 'differentiates'.

Minor intrusions

Steeply dipping north-south basaltic dykes that are approximately normal to the flows are found at all levels in the exposed section. Dyke density increases notably towards sea level reaching a dilation value of 10%, measured over a 1.6-km transect normal to the swarm, through the drill site. In spite of the obvious drawback in sampling near-vertical rock units with a vertical drill hole, the excellent core recovery allows dykes to be identified in the core on the basis of their chilled contacts,

their essentially non-vesicular character, and frequently by their large vertical extent. An example of the multiple dykes which occur is the particularly thick complex of dykes between 528 m and 586 m (units 90.1, 90.2, 90.3, 90.4, 91.2, 92.1, 94.1 and 94.2). There is a broad distribution of intrusive contact angles in the core. Over 40% of these contact angles lie between 55° and 75°. The remainder are rather uniformly distributed over all other angles. In total, 98 minor intrusions constitute 40% of the drill core; however, 11 of these, with vertical extent of greater than 20 m, account for over half of this percentage. The seven minor intrusions in the uppermost 400 m of the drill core comprise five isolated examples and one two-component multiple dyke of 110 m vertical extent (units 10.1 and 17.1, 63 to 173 m). Below 400 m the distribution of minor intrusions is markedly non-uniform. Minor intrusions are concentrated in three intervals each of about 300 m vertical extent separated by comparable intervals in which intrusions are generally much less common (Fig. 2b).

Preliminary observations suggest that the minor intrusions are generally less altered than the intervening flows. Thin-section studies indicate that at least some of the dykes in the core are olivine bearing and thus may not be directly related to the lavas of the drill core which are mainly olivine free.

Some of the minor intrusions occurring below 400 m in the drill core are almost certainly gently dipping sheets of a type characteristic of the deeper parts of many Icelandic Tertiary and Quaternary volcanic centres. There is no demonstrable increase in the proportion of minor intrusions as a whole with depth in the drill core (0–500 m, 34%; 500–1,000 m, 42%; 1,000–1,500 m, 45%; 1,500–1,919 m, 39%) or in the proportion of thick, apparently steeply dipping dykes.

Secondary mineralisation

A generally progressive increase of alteration with depth in the section characterises the lavas but is not obvious in the dykes. The topmost flows in the exposed section are unzeolitised. Zeolites appear lower in the exposed section (Fig. 2a). In general laumontite is absent in the exposed section, but is found in the Skessa tuff, a silicic ash-flow 100 m above the drill site.

All the flows in the drill core are variably altered, with macroscopic secondary minerals abundantly developed in the brecciated and vesicular flow tops and bottoms. Laumontite is dominant in the upper 1,000 m of the core. A shift from smectite to chlorite dominated assemblages occurs at the 200–300-m depth. Epidote first becomes very common at 1,200 m, where it occurs in both amygdalae and veins and in the groundmass of the

basalts. From 1,200 m to the bottom of the core at 1,919 m epidote is patchily distributed with alternating rich and deficient sequences. Calcite, various forms of silica and 'celadonite' are common secondary minerals throughout the drill core and various parts of the exposed section. Of particular interest are grossular garnet at dyke contacts at 425 and 1,166 m and anhydrite at 1,055 m.

The alteration pattern of the lavas in the drill core is very similar to that found in other drilled sections in the Tertiary formations of Iceland⁷.

Clearly the secondary mineralisation took place at a very much higher temperature than that measured in the hole at present. Based on data from drill holes in the active volcanic zone of Iceland, the lower boundary for the formation of laumontite seems to be about 110 °C and that for epidote about

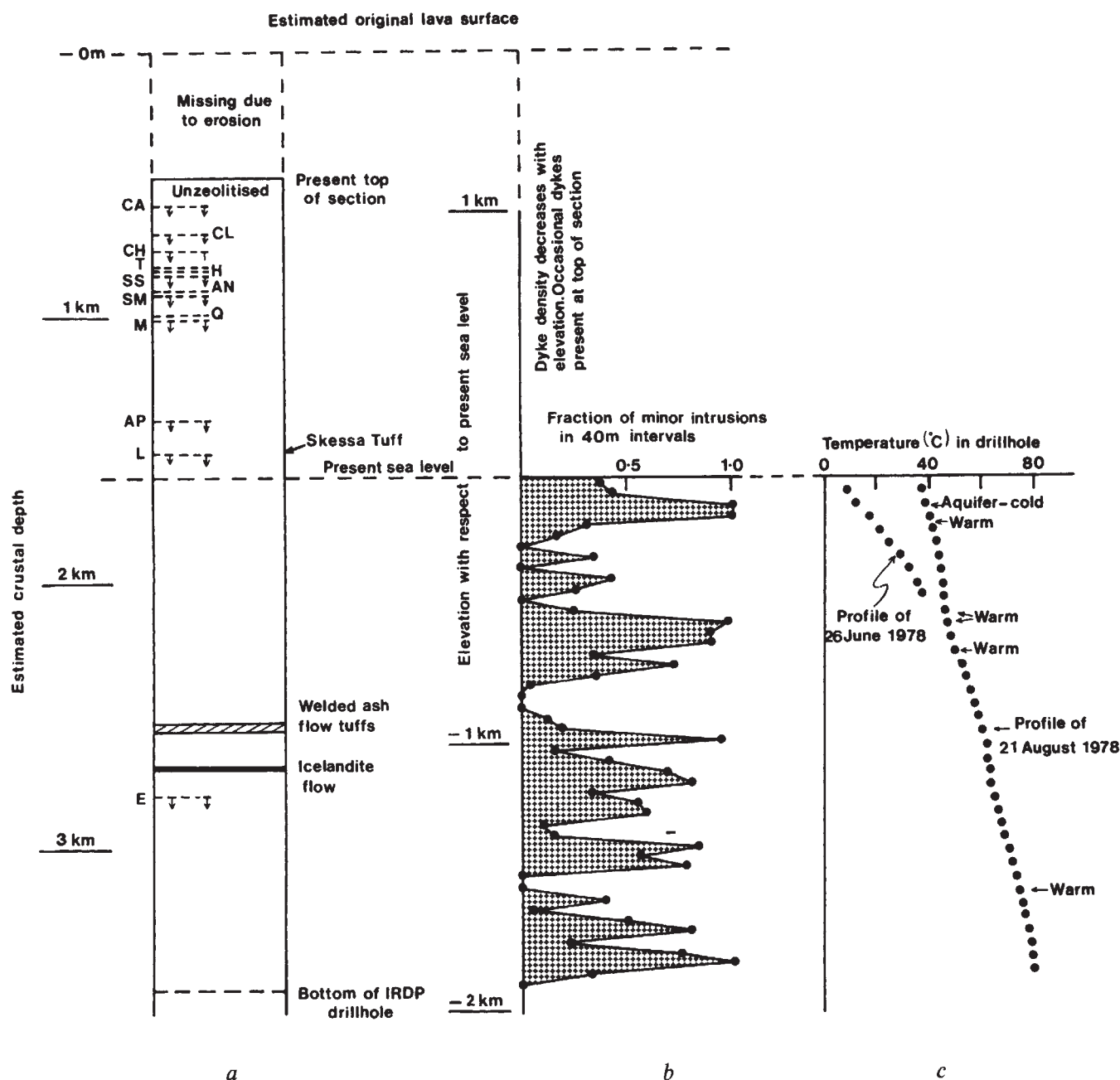


Fig. 2 Profiles through the 3-km Icelandic crustal section. *a*, Marker horizons and highest occurrence of key secondary minerals are indicated by a dashed line and downward pointing arrows. CA, calcite; CL, clay; CH, chabazite; T, thomsonite; H, heulandite; SS, stilbite; AN, analcite; SM, scolecite-mesolite; Q, quartz; M, mordenite; AP, apophyllite; L, laumontite; and E, epidote. *b*, Fraction of minor intrusions in each 40 m depth interval. *c*, Temperature profiles before and after penetration of the series of artesian warm water aquifers between 517 m and 628 m depth.

230 °C (ref. 8). Assuming an original surface 1,500 m above sea level and a linear temperature gradient, the figures suggest the existence of a temperature gradient of close to 85 °C km⁻¹ during the formation of the secondary mineralisation.

Magnetic properties

Measurements of initial susceptibility and polarity of natural remanence magnetism (NRM) were obtained for the lower 2 km of the section. Susceptibility values were obtained every 50 cm or less, correction being made for the size and cylindrical shape of the core. Although remanence cleaning is necessary to define the polarity succession reliably, natural remanence polarity measurements may be of some value in locating the section with respect to the known time-magnetic polarity sequence.

The most important feature of the susceptibility measurements is a strong and regular overall decrease with depth for the flows from a mean value of 40×10^{-4} e.m.u. cm⁻³ for the 0–480-m depth interval to 14×10^{-4} e.m.u. cm⁻³ for the 1,440–1,919-m interval. Linear extrapolation of this trend in mean values for the lavas suggests that susceptibility would reach zero value at 2,600 m below sea level or at just over 4 km beneath the original surface of the lava pile. In contrast, no depth trend in minor intrusion susceptibility occurs, with mean values for 480-m depth intervals varying irregularly from 26 to 31×10^{-4} e.m.u. cm⁻³. Susceptibility typically varies little within minor intrusions, except where fractures or intervals of high permeability occur. In contrast, strong within-unit variation is common for flows, with minima at boundaries and one or two interior maxima. In a general sense, susceptibility variation follows the alteration pattern: the downhole decrease in flow values matches the increase in alteration grade from a laumontite stage to an epidote stage while the minima at flow boundaries correspond to maximum secondary mineral development in these porous regions.

The relatively high average values, and lack of a trend with depth in minor intrusion susceptibility, corresponds with the apparent low alteration state of these units, and may also be indicative of their much younger age. Decomposition of the iron-titanium oxides resulting from zeolite and epidote grade metamorphism is likely to explain reduction of susceptibility in the flows.

Above 1,230 m depth only normal NRM polarity occurs. Reverse NRM polarities, including shallow inclinations, occur mainly within the following depth intervals: 1,445–1,635, 1,690–1,730 and 1,882–1,919 m. Multiple changes of NRM polarity within units have frequently been found in the lower bore-hole section.

At this stage the extended normal polarity zone of the upper half of the core apparently correlates with magnetic anomaly 5 (magnetic epoch 9).

Geothermal measurements

Temperature profiles in the drill hole were obtained weekly, in each case after a 24-h cessation of drilling. Initial water samples were taken from four levels several days after the termination of drilling, but these showed signs of mixing with drilling fluid and the sampling will be repeated. A temperature profile measured when the well was 445 m deep (Fig. 2c) showed an approximately linear thermal gradient of about 80 °C km⁻¹. Between 517 m and 628 m a series of prominent artesian aquifers with water at 48 °C were penetrated. These aquifers were tested when the hole was at a depth of 650 m and produced a net flow of 0.8 l s⁻¹. Several smaller aquifers were penetrated at greater depths. Flow of water from the prominent aquifers led to a major change in thermal regime in the hole (Fig. 2c); the temperature gradient below these aquifers is much lower.

The site was located within an area of anomalously high surface temperature gradient, and results from the drill hole strongly suggest that this anomalous area is the result of shallow regional geothermal flow of water at moderate temperatures.

Geophysical logging

Extensive geophysical logging of the drill hole was carried out by the Icelandic National Energy Authority with natural γ -ray, neutron-neutron porosity, γ - γ density, caliper, self-potential, 0.4 m and 1.6 m normal resistivity, temperature, differential temperature, sonic velocity and differential magnetic permeability recorded. In the cased central part of the hole (580–1,350 m) self-potential, resistivity and differential permeability could not be recorded. Sonic velocity was recorded from the surface to 1,350 m depth.

Preliminary results show a very pronounced relationship between the physical and lithological parameters. An example is the high resistivity contrast between the massive centres of lava flows and the brecciated or rubbly margins. Dykes and intrusions are also characterised by high resistivity. Relatively few, but prominent, anomalies in the natural γ -ray log are recorded. The sequence of welded ash-flow tuffs between 920 and 951 m depth is characterised by high natural radioactivity and low γ - γ density. Thin breccia layers can be identified in the neutron-neutron porosity log as well as in the γ - γ density log.

Crustal seismology

Two crustal seismic experiments were carried out in association with the drilling project, one by a group from Leningrad and the other by a group from the University of Washington and the Scripps Institution of Oceanography. The results from these experiments are still being interpreted.

Discussion

Although intensive laboratory studies of the 3-km section are continuing, some important points are already evident. Perhaps the most striking feature is the continuously sub-aerial nature of the lavas implying that at least 2 km of subsidence has occurred. No pillowed basalts were found. The absence of marked erosional features in the exposed upper part of the section suggests that the lavas were erupted in an area of low relief at most a few hundred metres above sea level and the implication is that subsidence probably took place concomitant with the volcanism at a rate of at least 1 km Myr⁻¹. Subsidence on this scale is implied in many of the models for crustal growth in Iceland (see ref. 9) and is observed elsewhere where active, volcanic zones occur in areas of oceanic crust^{10–12}.

The progressive change in the nature of the alteration with depth is also striking. The intensity of metamorphism in the lavas increases gradually, but in detail relatively fresh rocks interfinger with more altered horizons. If the layer 2/layer 3 boundary is fundamentally a metamorphic transition then the present study suggests that such a transition may take place over a distance of the order of 1 km.

The combination of the olivine-bearing nature of some of the minor intrusions, the magnetic properties, and the relative freshness of most of them may imply that they were not feeders for the bulk of the lava succession. The possibility that the dykes are related to the Breiddalur centre¹³, 20 km to the south, and that they have been injected laterally northwards in the manner that is presently being observed in the Krafla volcano in north Iceland¹⁴ must be considered.

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Isolation, cloning and sequence analysis of the cDNA for the α -subunit of human chorionic gonadotropin

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A 621-base pair fragment of the cDNA for the α -subunit of human chorionic gonadotropin has been isolated by cloning in a plasmid vector, and the complete nucleotide sequence determined. The entire coding region, including the 24-amino acid pre-sequence and most of the untranslated regions, are present in the fragment.

HUMAN chorionic gonadotropin (HCG) is a glycoprotein hormone which is synthesised by the syncytiotrophoblast cells of the placenta. It functions during pregnancy to convey a signal from the placenta to the corpus luteum which results in the maintenance of the corpus luteum and an increased synthesis of steroid hormones (for reviews see refs 1, 2). Certain malignant tumours, not necessarily of placental tissue, also synthesise HCG^{3,4}. Detection of HCG by radioimmunoassay is therefore diagnostic of pregnancy or the presence of a tumour⁵.

In addition to its medical importance, HCG is an interesting system for studying temporal gene regulation during development. Levels of HCG in the urine can be detected a few days after implantation of the fertilised ovum⁶. Production of the hormone reaches a peak level of about $1 \mu\text{g ml}^{-1}$ in the plasma at 10-12 weeks and then declines considerably during the second and third trimesters⁷. It is not understood what controls this rise and decline of HCG synthesis during pregnancy nor why such large amounts of the hormone are synthesised towards the end of the first trimester.

The three anterior pituitary hormones, luteinising hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH), are closely related to HCG in that all four hormones are glycosylated and have a dimeric structure comprising an α - and a β -subunit^{1,2,8}. The amino acid sequence of the α -subunit, which is 92 residues long, seems to be identical in all four hormones⁹⁻¹⁵ whereas the amino acid sequences of the β -subunits vary^{10,16-22}. The β -subunits confer the biological specificity to the hormones^{1,2}. Sequence homologies exist between the β -subunits, and to a lesser extent between the β -subunits and the α -subunit, leading to the proposal that all five subunits evolved from a common ancestral gene²³.

The glycoprotein hormones therefore provide an interesting group of structurally related sequences which have been studied extensively in terms of their protein chemistry and biological functions but for which very limited information is available about their mRNA and genomic organisation. This report describes the isolation, by cloning in pBR322, of a 621-base pair

cDNA fragment synthesised from first-trimester placental polyadenylated mRNA. The fragment contains the coding sequences for the 92 amino acids of the α -subunit of HCG, the 24 amino acid pre-sequence and most of the 5'- and 3'-untranslated regions.

In vitro translation of placental RNA

Full-term and first-trimester placental mRNA has been analysed by *in vitro* translation and cDNA synthesis to determine whether the higher levels of HCG produced by first-trimester placentae are reflected by higher amounts of cDNA than occur in full-term placentae.

The major protein product synthesised by full-term placenta is the hormone human chorionic somatomammotropin (HCS). Full-term placental polyadenylated mRNA has been analysed previously by wheat-germ translation and immunoprecipitation of the translation products with an antiserum against HCS²⁴. The predominant translational product was a protein of molecular weight (MW) 24,000, which contained tryptic peptides in common with HCS²⁵, and which was precipitated specifically by the antiserum and thus identified as the pre-form of HCS. A similar approach has been used here to analyse first-trimester placental polyadenylated mRNA, which should be enriched for HCG-specific mRNA. The *in vitro* translation products have been compared with those obtained from full-term mRNA, which should have much less HCG-specific mRNA.

DNA was extracted from human placenta obtained either at term (from caesarian sections) or from first-trimester abortions (about 10-12 weeks). The RNA samples were translated in a wheat-germ cell-free translation system with ³⁵S-methionine and the products fractionated on an SDS-15% polyacrylamide gel (Fig. 1). Full-term placental RNA (lane 3) shows the major product of MW 24,000 which has been identified as pre-HCS^{24,25}. This product is still observed with first-trimester placental RNA (lane 2) but at a considerably lower level. This is consistent with the observations made by Boime *et al.*²⁵ and by Chatterjee *et al.*³¹, who have also compared the synthesis of HCS by full-term and first-trimester RNA in cell-free translation systems.

The major proteins synthesised *in vitro* by first-trimester RNA (lane 2) have estimated MWs of 13,000 and 16,000 and are only observed in trace quantities with full-term RNA (lane 3). These products are candidates for the pre-forms of the α - and β -subunits of HCG, respectively, as the respective MWs of the

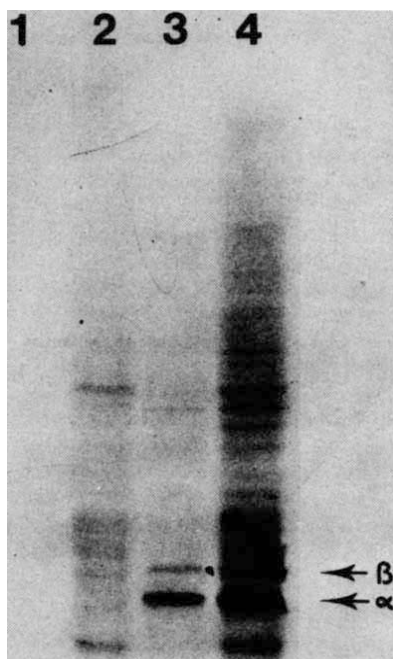


Fig. 1 Wheat-germ translation products of full-term and first-trimester placental mRNA. Human placental tissue was obtained from either caesarian sections or first-trimester abortions. The tissue was washed in cold saline to remove blood, frozen quickly in liquid nitrogen and stored at -70°C . RNA was prepared by methods similar to those described previously^{24,26}. Ten grammes of frozen tissue were added to 100 ml of 5 M guanidine thiocyanate in 50 mM Tris-Cl, pH 7.5, 10 mM EDTA and 5% 2-mercaptoethanol and homogenised in a Tissumizer (Tekmar) until all the tissue was dispersed. The solution was centrifuged at 10,000 r.p.m. for 10 min and the supernatant adjusted to 2% Sarkosyl and heated at 65°C for 2 min. Caesium chloride was then added (0.1 g ml^{-1} of solution) and the resulting solution was layered over 6-ml

cushions of half-saturated CsCl in 10 mM EDTA in SW 27 cellulose nitrate tubes. Centrifugation was at 25,000 r.p.m. for 16 h at 20°C . The RNA pellet was dissolved in 5 mM EDTA, 0.5% Sarkosyl and 5% 2-mercaptoethanol, extracted with phenol and chloroform and precipitated with ethanol. Usually, 0.5–1 mg of RNA were obtained per g of tissue. The RNA was then passed over an oligo(dT)-cellulose column²⁷ to enrich for the polyadenylated fraction. RNA samples were translated by wheat-germ cell-free extracts^{28,29}. Reaction mixtures (40 μl) contained 20 mM HEPES buffer, pH 7.6, 2 mM dithiothreitol, 0.1 mM spermidine, 80 mM K acetate, 1 mM Mg acetate, 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate, 40 $\mu\text{g ml}^{-1}$ creatine phosphokinase, $\sim 25\text{--}50\text{ }\mu\text{Ci }^{35}\text{S}$ -methionine (400–900 Ci mmol⁻¹, Amersham), 50 μM each of the other 19 amino acids, 16 μl of wheat-germ extract and 0.5–1.0 μg polyadenylated RNA. Incubation was at 28°C for 1 h. The products were analysed on an SDS-15% polyacrylamide slab gel³⁰ and the dried gel autoradiographed. Lane 1, endogenous wheat-germ products; lane 2, first-trimester placental RNA; lane 3, full-term placental RNA. The proposed identifications of the translational products, as discussed in the text, are indicated.

maturation of these proteins, without glycosylations, are 10,200 and 15,500 (refs 9, 10, 16–18).

The wheat-germ products synthesised in the presence of first-trimester RNA were immunoprecipitated by an antiserum directed against mature HCG and the products fractionated on an SDS-15% polyacrylamide gel (Fig. 2). The translational products generated by first-trimester RNA are shown in lane 4 and the immunoprecipitated products in lane 3. Both the 13,000 and 16,000 MW products seem to precipitate specifically, supporting their identification as pre-forms of the α - and β -subunits of HCG, respectively. In general, however, the immunoprecipitation reactions were inefficient and unreliable. The weak antibody-antigen reaction is presumably related to the fact that the antibody was raised against the mature glycosylated dimeric form of HCG and not against the non-glycosylated pre-forms of the individual subunits which are produced in cell-free translation systems. A small amount of pre-HCS is also precipitated in this reaction (lane 3) but this seems to be the result of a specific reaction between HCS and the formaldehyde-treated *Staphylococcus aureus* cells used to collect the antigen-antibody complex, because it also precipitates in the absence of antibody but in the presence of the *S. aureus* cells.

The data presented here are consistent with other results. It is reasonable to expect a greater quantity of α - than β -specific mRNA as there is an excess amount of free α -subunit over free β -subunit in plasma³³. Also, Landefeld *et al.*³⁴ have shown that the major translational product from first-trimester RNA has tryptic peptides in common with the native α -subunit. Daniels-

McQueen *et al.*³⁵ and Chatterjee *et al.*^{31,36} have observed lower levels of β -subunit synthesis than α -subunit synthesis in cell-free systems and have demonstrated a greater amount of synthesis of these subunits with first-trimester than with full-term RNA.

Restriction enzyme analysis of placental cDNA

Discrete restriction endonuclease fragments can be generated from heterogeneous cDNA if that cDNA contains a reasonably enriched species. This approach has been used to clone fragments of human growth hormone, HCS, rat growth hormone and rat insulin cDNAs^{37–40}. The differences in translational activities observed between first-trimester and full-term human placental RNA should therefore also be observed in the restriction enzyme fragments generated from cDNA synthesised from these two sources of mRNA. Comparison of the two cDNA fragment patterns should allow identification of fragments in first-trimester cDNA which probably correspond to either α - or β -HCG and thus facilitate cloning of these cDNA species.

Double-stranded cDNA was synthesised from polyadenylated RNA obtained from both first-trimester and full-term placentae and was restricted with either *AluI* or *HpaII*. The products were fractionated by electrophoresis on a 5% polyacrylamide gel (Fig. 3). With both enzymes a specific band pattern is observed which varies with the RNA used to generate the cDNA. The 340-base pair fragment obtained when full-term cDNA is digested with *AluI* (lane 1) is known from sequence analysis to be present in HCS cDNA³⁸ and is only observed at a very low level in first-trimester cDNA (lane 2). In contrast, *AluI* digestion of first-trimester cDNA (lane 2) generates fragments

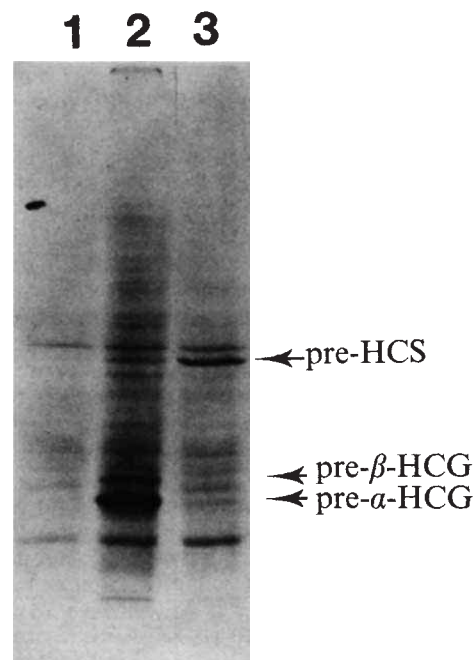


Fig. 2 Immunoprecipitation of wheat-germ products by HCG antiserum. The wheat-germ translation products generated by first-term placental RNA (Fig. 1) were immunoprecipitated by an antiserum directed against native HCG and the antigen-antibody complex collected by formaldehyde-treated *S. aureus* cells^{29,32}. The products were fractionated on an SDS-15% polyacrylamide gel³⁰ and visualised by autoradiography of the dried gel. Lane 1, immunoprecipitation of endogenous wheat-germ products; lane 2, endogenous wheat-germ translation products; lane 3, immunoprecipitation of wheat-germ translation products directed by first-trimester placental polyadenylated RNA; lane 4, wheat-germ products of first-trimester placental polyadenylated RNA. The proposed identifications of the pre-forms of the α - and β -subunits of HCG are indicated.

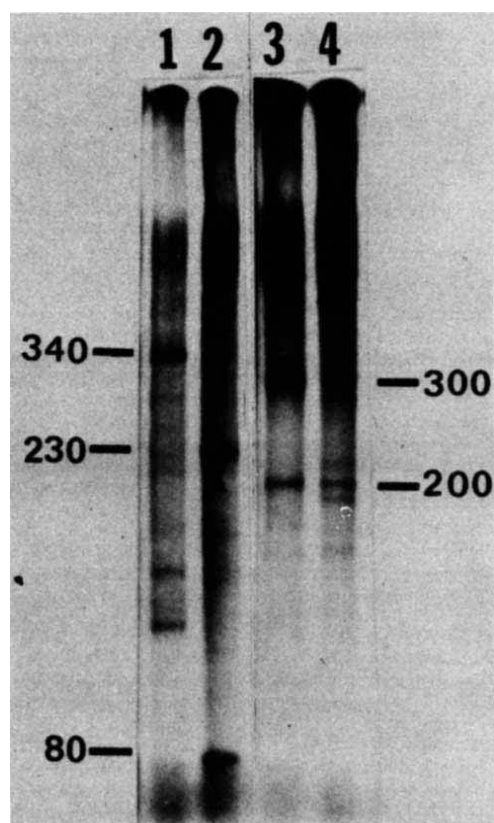


Fig. 3 Restriction enzyme analysis of double-stranded cDNA made from polyadenylated RNA extracted from both full-term and first-trimester human placenta. The methods used for synthesis of double-stranded cDNA were similar to those described previously^{37,39,41}. For preparative syntheses, ~20 µg polyadenylated RNA were incubated in 200 µl reactions containing 50 mM Tris-Cl, pH 8.3, 10 mM MgCl₂, 20 mM KCl, 10 mM 2-mercaptoethanol, 500 µM each of dATP, dCTP, dGTP and dTTP (one [α-³²P]-nucleoside triphosphate at specific activity 500 c.p.m. ³²P per pmol), 20 ng µl⁻¹ oligo(dT)₁₂₋₁₈ and 200 units reverse transcriptase. Incubation was at 42 °C for 30 min and was stopped by the addition of EDTA to 10 mM. After extraction with phenol and chloroform and precipitation by ethanol, the material was fractionated on a 25 × 0.25 cm column of Sephadex G-50 in 1 mM Tris-Cl, pH 7.4, and 0.01 mM EDTA. The leading peak was collected (~200 µl) and adjusted to 0.3 M NaOH and 10 mM EDTA and incubated overnight at room temperature. Following neutralisation with sodium acetate, pH 5.5, and ethanol precipitation, the second strand was synthesised in a 50-µl reaction containing the same buffer as described above, 500 µM each of dATP, dGTP and dTTP and 100 µM dCTP (10,000 c.p.m. ³²P per pmol) (considerably higher specific activities were used for analytical reactions) and 50 units reverse transcriptase. Incubation was at 42 °C for 90 min. For subsequent restriction enzyme analysis the reaction was stopped by heating to 65 °C for 10 min and the appropriate restriction enzymes added directly. Alternatively, the reaction products were fractionated as described above on a Sephadex G-50 column and the peak evaporated to dryness before proceeding to treatment with S₁ nuclease (see Fig. 4 legend). Lane 1, *AluI* digest of full-term placental cDNA; lane 2, *ALuI* digest of first-trimester placental cDNA; lane 3, *HpaII* digest of full-term placental cDNA; lane 4, *HpaII* digest of first-trimester placental cDNA. Sizes are in base pairs.

of 230 and 80 base pairs that are not observed in full-term cDNA. The enzyme *HpaII* also generates different fragments from full-term and first-trimester cDNAs (lanes 3, 4). It is reasonable to assume that the fragments specific to first-trimester cDNA correspond to either α- or β-HCG cDNA.

Cloning of the cDNA for the α-subunit of HCG

The comparisons of the wheat-germ translation products and the cDNAs synthesised from full-term and first-trimester placental polyadenylated mRNAs suggest that it should be possible to clone the HCG cDNA species from first-trimester material without any further enrichment procedures.

Double-stranded cDNA was synthesised by reverse transcriptase from 20 µg of first-trimester polyadenylated mRNA^{37,39,41} (Figs 3, 4). Yields of about 5% and 25% were obtained on the first- and second-strand syntheses, respectively. An aliquot of the double-stranded cDNA was electrophoresed on a 5% polyacrylamide gel and the products visualised by autoradiography (Fig. 4, lane 2). A prominent band corresponding to a chain length of about 700 base pairs can be seen above the heterogeneous cDNA background. This species was also observed, but with the expected slower mobility, in the single-stranded cDNA (Fig. 4, lane 1). Presumably, this species corresponds to a full-length cDNA for either the α- or β-subunit of HCG, as 700 base pairs has sufficient coding capacity for either subunit plus untranslated regions, but not for the two together. Cleavage of the double-stranded cDNA with the enzyme *HindIII* generates a new band (about 630 base pairs) plus a residual low level of the 700-base pair band (Fig. 4, lane 4). As this was observed reproducibly it is not believed to be the result of partial digestion. Instead, it is interpreted to mean that the initial undigested 700-base pair band contains two cDNA species, presumably corresponding to the α- and β-subunits of HCG, and that the major species of cDNA contains a *HindIII* site about 50 base pairs from one end.

The full-length cDNA in the size range 600–750 pairs was then cloned into the unique *HindIII* site of pBR322 using synthetic *HindIII* linkers as described in Fig. 4 legend. Small plasmid DNA preparations were made from the 22 recombinants obtained in the transformation and analysed by restriction enzyme digestion. The enzymes *PstI* and *XbaI* were used because first-trimester cDNA cleaved with these enzymes produces specific fragments, about 230 base pairs long in the case of *XbaI* and about 380 and 170 base pairs long in the case of *PstI* (Fig. 4, lanes 7, 8). These fragments are presumably diagnostic of HCG cDNA and can be readily detected, as the plasmid pBR322 contains a single *PstI* cleavage site and no *XbaI* cleavage site⁴⁴. One recombinant was obtained; it had a *HindIII* insert about 620 base pairs long which when digested with *XbaI* gave fragments of 400 and 220 base pairs and when digested with *PstI* gave fragments 370, 170 and 80 base pairs long. In addition, this 620-base pair *HindIII* fragment contains *AluI* and *HpaII* fragments consistent with those observed in first-trimester cDNA (Fig. 3). This recombinant cDNA clone was thus considered a candidate for the α-subunit of HCG because it corresponds to the major cDNA species. No candidates for β-HCG cDNA have been obtained.

DNA sequence analysis of α-HCG cDNA

DNA sequencing was used to show that the *HindIII* fragment codes for the α-subunit of HCG and is 621 bases long. Initial identification of the recombinant, designated α-HCG/pBR322, was by a modification of the sequencing technique described by Maat and Smith⁴⁵ for sequencing 5'-terminally labelled DNA fragments by the chain termination method (A. J. H. Smith, personal communication). The end-labelled DNA fragments were incubated with DNA polymerase I (Klenow modification) and a single dideoxynucleoside triphosphate, and the products were loaded directly onto a DNA sequencing gel (see Fig. 5 legend). This method results in the introduction of the chain terminators into the double-stranded DNA, presumably at the sites of nicks in the DNA caused by the contaminating DNase in DNA polymerase I preparations. This was found to be a particularly rapid method for analysing potential recombinants, but difficulties were encountered in obtaining accurate sequences where there were stretches of a single nucleotide.

The complete nucleotide sequence of the recombinant was obtained following transfer of the 621-base pair *HindIII* fragment to the single-stranded DNA phage vector M13 mp5. Standard chain termination methods were used^{46,48} and, where possible, sequences were obtained on both strands, but in all cases the results were confirmed by additional primings. The complete 621-base pair sequence is presented in Fig. 5.

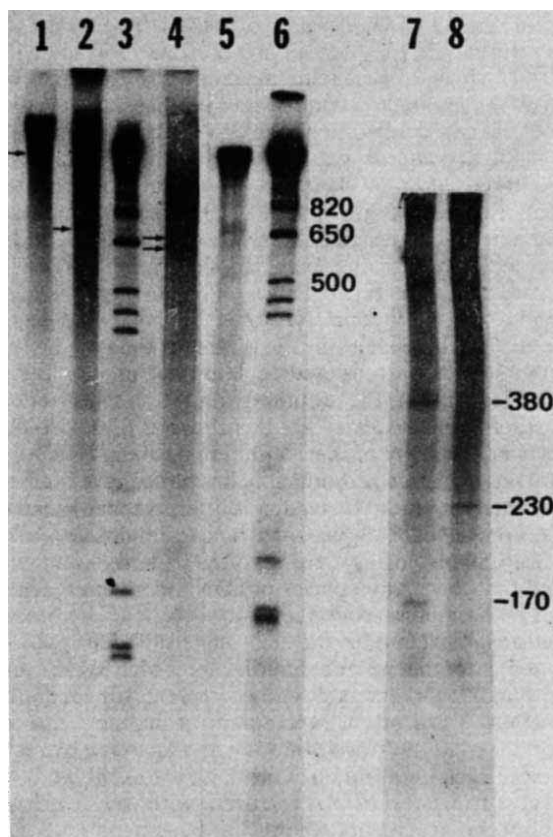


Fig. 4 Synthesis and cloning of first-trimester cDNA. The methods used for cloning the cDNA using *Hind*III linkers were similar to those described elsewhere^{37,39}. Synthesis of double-stranded cDNA from 20 µg polyadenylated RNA was as described in Fig. 3 legend. After fractionation by Sephadex G-50, the double-stranded cDNA was incubated with 5 units of *S*₁ nuclease in 300 mM NaCl, 30 mM Na acetate, pH 4.5, and 3 mM ZnCl₂ at 42 °C for 5 min (refs 37, 41). The reaction was stopped by the addition of EDTA to 10 mM and neutralised with Tris base. Following extraction with phenol and chloroform, the products were fractionated over a Sephadex G-50 column (Fig. 3) and the peak fraction evaporated to dryness. The DNA was then incubated in a 25-µl reaction with 50 mM Tris-Cl, pH 7.5, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, 25 µM each of dATP, dCTP, dGTP and dTTP and 1 unit of DNA polymerase I (Klenow fragment, Boehringer) at 12 °C for 15 min. The reaction was adjusted to 50 µl in the same buffer with 1 mM ATP and 12 µg kinase-treated [5'-³²P]-labelled *Hind*III decanucleotide linkers (2×10^6 c.p.m. total) and incubated with 0.3 units T4 DNA ligase and 2.5 units T4 RNA ligase (Biolabs) at 12 °C overnight. After heating the reaction to 65 °C for 10 min, 50 units *Hind*III were added directly and the reaction was incubated at 37 °C for 2 h. Aliquots of the reaction were assayed at all stages by electrophoresis on 5% polyacrylamide gels and autoradiography of the dried gels. Following extraction with phenol and chloroform, the cDNA, with cohesive *Hind*III ends, was fractionated on a preparative 5% polyacrylamide gel and fragments in the size range 600–750 base pairs recovered by electrophoretic elution. The cDNA was then ligated to 100 ng *Hind*III-cleaved, alkaline phosphatase-treated pBR322 (refs 37, 39, 42) and used to transform *E. coli* χ 1776 as described elsewhere⁴³. Transformation and subsequent growth were carried out in a P3 facility in compliance with the NIH Guidelines for Recombinant DNA Research. Twenty-two colonies were obtained which were resistant to 20 µg ml⁻¹ ampicillin and sensitive to 20 µg ml⁻¹ tetracycline. The recombinants contained *Hind*III inserts of the appropriate size and were analysed by restriction enzyme digestion, in particular with *Pst*I and *Xba*I (see text). Lane 1, full-length single-strand cDNA; lane 2, full-length double-strand cDNA; lane 3, *Hpa*II digest of M13 DNA; lane 4, double-stranded cDNA digested with *Hind*III; lane 5, double-stranded cDNA digested with nuclease *S*₁; lane 6, as lane 3; lane 7, *Pst*I digestion of double-stranded cDNA; lane 8, *Xba*I digestion of double-stranded cDNA. Lanes 1–4, 5–6 and 7, 8 are from separate gels. Autoradiographs were made from dried 5% polyacrylamide gels. Sizes are in base pairs.

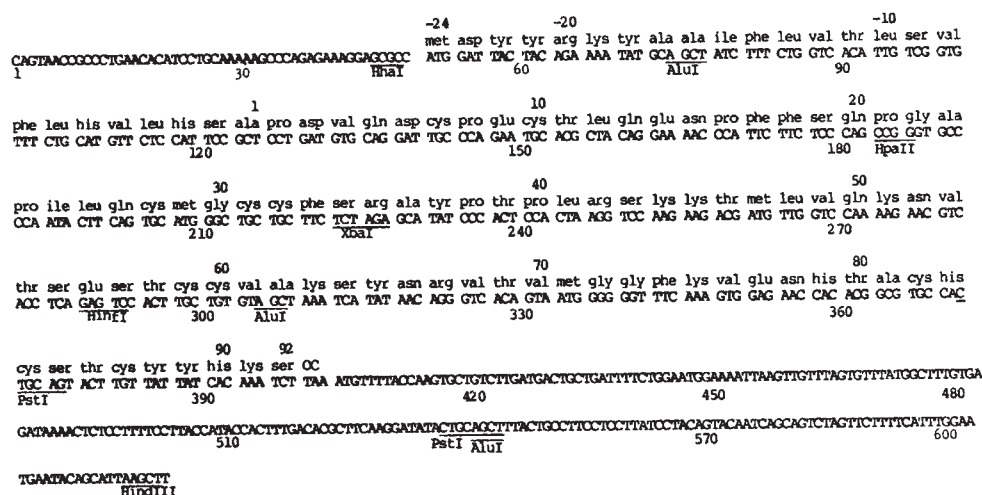


Fig. 5 Nucleotide sequence of the 621-nucleotide-long cDNA fragment coding for the α -subunit of HCG. The amino acid sequence of the 92-residue-long mature protein is shown as well as the 24-residue pre-sequence deduced from the cDNA sequence. Nucleotides are numbered below the sequence and amino acids above the sequence. The restriction endonuclease sites used in the sequence analysis are indicated. DNA from α -HCG/pBR322 was cleaved at the unique *Xba*I site, labelled with [γ -³²P]ATP by polynucleotide kinase and then digested with *Hind*III. The 400-base pair *Hind*III-*Xba*I fragment was isolated and divided into four separate 5-µl reactions each containing 6 mM Tris-Cl, pH 7.4, 6 mM MgCl₂, 1 mM dithiothreitol, 0.2 mM of a single dideoxynucleoside triphosphate and 1 unit DNA polymerase I (Klenow modification) and incubated at 37 °C for 1 h (ref. 45 and A. J. H. Smith, personal communication). The products were analysed directly on thin sequencing gels⁴⁶. After the 621-base pair fragment had been shown by this method to code for α -HCG, it was transferred to the unique *Hind*III site of the single-stranded filamentous DNA phage vector M13 mp5. This phage is a derivative of M13 mp2 (ref. 47); its transformation and growth (J. Messing, unpublished results) were done in a P3 facility in accordance with the NIH Guidelines for Recombinant DNA Research. Initially, the host strain 71/18 was used but has lately been replaced by JM101 (J. Messing, unpublished results). Both the coding and non-coding strands of the cDNA were inserted separately into the viral strand of the phage DNA and were used as templates for DNA sequencing reactions by the chain termination method⁴⁸. Restriction enzyme fragments for use as primers were obtained from α -HCG/pBR322.

The finding that the α - and β -subunits of the glycoprotein hormones are synthesised from separate polyadenylated mRNA species agrees with the deduction made by Daniels-McQueen *et al.*³⁵, who observed that the α - and β -translational activities for HCG sedimented at different positions in sucrose gradients.

The amino acid sequence for the 92 codons of the mature form of α -HCG as determined previously^{9,10} corresponds to that predicted from the DNA sequence (Fig. 5) with the exception of codon 15. Morgan *et al.*¹⁰ identified this position as aspartic acid whereas Bellisario *et al.*⁹ did not distinguish between aspartic acid or asparagine. DNA sequence analysis shows that the correct amino acid is asparagine. This assignment agrees with the sequences reported for the α -subunits of human LH and FSH^{11,12,14} which are probably identical to α -HCG. The sequences reported for the α -subunits of HCG^{9,10} and human FSH¹⁴ have the sequence Cys-Ser at positions 84 and 85, whereas this order is reserved in the reported sequence for human α -LH¹¹. The DNA sequence analysis of α -HCG cDNA supports the former identification of this dipeptide. N-terminal heterogeneity has been reported for the α -subunits of human glycoprotein hormones involving the first three amino acids and was assumed to be the result of proteolytic cleavage. The identification of the coding sequence for this region supports this assumption.

A pre-sequence of 24 amino acids can be predicted from the cDNA sequence (Fig. 5) and has the same characteristic hydrophobic stretches observed in other pre-sequences (see ref. 49). This sequence agrees with the preliminary results of Boime *et al.*⁵⁰, who have identified a pre-sequence of 24 amino acids for the α -subunit of HCG and have located the positions of the Ala, Phe and Leu residues.

The coding region ends with the termination codon UAA and is followed by 220 bases to the *Hind*III site. This restriction enzyme site is not the result of a linker ligation but corresponds to the natural *Hind*III site observed in the cDNA. Approximately 50 bases of the 3'-terminal region of the cDNA are thus absent from the clone, including the sequence AAUAAA which is characteristic of all polyadenylated eukaryotic mRNA species⁵¹. Fifty bases of the 5'-untranslated region are present in the clone before the *Hind*III linker molecule. The sequence AAGGAG located at positions -10 to -5 before the initiator AUG shows perfect complementarity with the 3'-end of *Escherichia coli* 16S rRNA and is thus similar to a prokaryotic ribosome binding site⁵².

The codon usage observed with the α -subunit of HCG, including the pre-sequence, is shown in Fig. 6. Of the 10 serine codons, nine are of the form UCN and only one is AGPy; all four arginine codons are of the form AGPu and none are CGN.

The similarity in amino acid sequence among all four α -subunits for human CG, LH, FSH and TSH suggests that they

2/UUU/phe	2/UCU/ser	5/UAU/tyr	2/UGU/cys
4/UUC/phe	4/UCC/ser	2/UAC/tyr	8/UGC/cys
0/UUA/leu	2/UCA/ser	1/UAA/OC	0/UGA/OP
2/UUG/leu	1/UCG/ser	0/UAG/AM	0/UGG/trp
1/CUU/leu	1/CCU/pro	2/CAU/his	0/CGU/arg
1/CUC/leu	1/CCC/pro	3/CAC/his	0/CGC/arg
2/CUA/leu	4/CCA/pro	1/CAA/gln	0/CGA/arg
2/CUG/leu	1/CGG/pro	4/CAG/gln	0/CGG/arg
0/AUU/ile	3/ACU/thr	0/AAU/asn	1/AGU/ser
1/AUC/ile	1/ACC/thr	4/AAC/asn	0/AGC/ser
1/AUA/ile	2/ACA/thr	4/AAA/lys	2/AGA/arg
4/AUG/met	3/ACG/thr	3/AAG/lys	2/AGG/arg
1/GUU/val	3/GCU/ala	3/GAU/asp	2/GGU/gly
4/GUC/val	1/GCC/ala	0/GAC/asp	1/GGC/gly
2/GUA/val	2/GCA/ala	2/GAA/glu	0/GGA/gly
3/GUG/val	1/GCG/ala	2/GAG/glu	1/GGG/gly

Fig. 6 Codon usage of α -HCG mRNA.

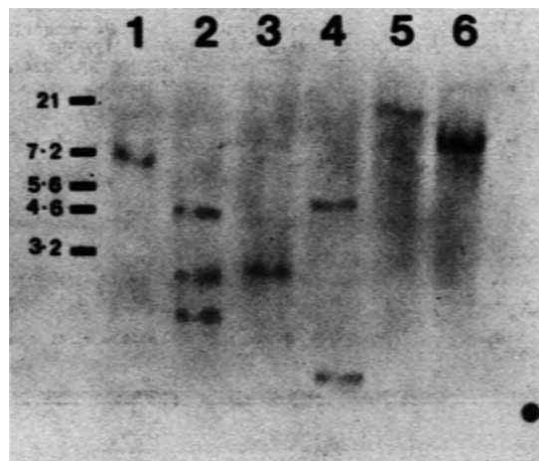


Fig. 7 Autoradiograph of a filter hybridisation between restriction endonuclease-digested human placental DNA, fractionated on a 1% agarose gel, and ³²P-labelled, cloned α -HCG cDNA. The filter hybridisation method of Southern⁵³ was used as described previously⁵⁴. Lane 1, *Eco*RI; lane 2, *Xba*I; lane 3, *Pst*I; lane 4, *Hind*III; lane 5, *Bgl*II; lane 6, *Pvu*II. The positions and sizes in kilobase pairs of the fragments produced by an *Eco*RI digest of wild-type bacteriophage λ DNA are shown.

are coded for by a single gene. To test this, the 621-base pair fragment was labelled with ³²P and used as a probe to hybridise to endonuclease-digested human placental DNA by the method of Southern⁵³ (Fig. 7). With three enzymes, *Eco*RI (lane 1), *Bgl*II (lane 5) and *Pvu*II (lane 6), single hybridising bands are observed. The *Eco*RI fragment is about 6.5 kilobases long. These results suggest the existence of either a single gene or multiple copies arranged in tandem within the 6.5-kilobase fragment.

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letters

Diffusion of the highest energy cosmic rays from Virgo

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The origin of the predominant particle component of cosmic rays is still undecided although at 'low' energies ($E \sim 10^9$ – 10^{10} eV) γ -ray data provide circumstantial evidence in favour of an origin in galactic sources¹. The galactic magnetic field deflects trajectories, at least until about 10^{18} eV, and almost completely destroys any relationship between the directions from which cosmic rays arrive and the directions to their sources, making the elucidation of origins a problem. Above 10^{18} eV, if the primary cosmic rays are mainly protons, as would be expected and as is claimed², then the absence of large anisotropies associated with the galactic plane strongly suggests that the particles are from extragalactic sources. If they are extragalactic and their sources are distributed widely in the Universe then, because of interactions between the protons and the photons of the 2.7 K relic radiation, the observed spectrum should steepen considerably above several times 10^{19} eV. However, the opposite appears to be happening: the measured spectrum flattens above about 10^{19} eV (refs 4–6) (see Fig. 2). Several suggestions have been made to account for this paradox, in each case the idea being to generate in extragalactic space a production spectrum which is so 'flat' that even after energy losses are taken into account, the resulting spectrum at the Earth has the characteristic observed flattening (for example ref. 7). Here we put forward an alternative model where the observed spectral shape arises in a rather natural way, being due to propagation characteristics in the local supercluster, the bulk of the very energetic particles having originated in its central region.

Many studies have been made of the diffusion of cosmic rays within the Galaxy but little attention has been given to the possibility of propagation in extragalactic space being by diffusive rather than rectilinear motion. This is due to lack of knowledge of the topography of the magnetic field in space. Recent measurements of X-ray emission from clusters of galaxies (some of which seems to be due to thermal bremsstrahlung from hot intergalactic gas) make us believe, however, that

significant magnetic fields exist. For example, if some measure of equipartition exists between field energy ($B^2/8\pi$) and kinetic energy of gas clouds ($\rho v^2/2$) then the likely gas densities of $\approx 10^{-4}$ atoms cm^{-3} in clusters indicate fields of $\approx 10^{-8}$ G.

A necessary parameter for particle diffusion is the cell length over which the field can be regarded as uniform—rough arguments suggest that a dimension of the order of a few galactic diameters (say 0.1 Mpc) might be reasonable. These arguments are: (1) A likely source of at least some of the cluster field is clouds of gas containing cosmic rays which are forced out of the galaxy when supernovae explode. Such clouds, with closed field loops, would presumably have dimensions of a few kpc on emergence from the galaxy and would expand at greater distances. (2) Within the galaxy the cell length seems to be about one hundredth of the mean linear dimension of the galaxy. One hundredth of the characteristic dimension of the supercluster would correspond to a few hundred kpc. There is, of course, no certainty about the magnitude of the cell length adopted, but it does seem as though a value within a factor 10 either way of 100 kpc is likely.

The Larmor radius of a proton of energy E_{20} (in units of 10^{20} eV) in a field of 10^{-8} G is $E_{20}/10$ Mpc, so diffusion might be expected to be very important for particles from all extragalactic sources at energies below $\approx 10^{18}$ eV and for particles from sources within some tens of Mpc with energy below 10^{20} eV or so. Above a few times 10^{21} eV there will be rectilinear propagation from sources within tens of Mpc.

The attenuation length for protons on the microwave background is well known. This length, λ , equals the distance to the centre of Virgo (19 Mpc) at $E_0 \approx 3 \times 10^{20}$ eV. The next 'large' cluster, Pegasus I, is at 65 Mpc, for which $E_0 \approx 1.3 \times 10^{20}$ eV. If the sources of particles of energy $\approx 10^{20}$ eV are only present in cluster centres, then these particles will only arrive from Virgo, particles from further afield having had insufficient time to travel to Earth. It is this feature of depressing cosmic rays from sources beyond Virgo that distinguishes the present model from previous ones (for example, the supercluster enhancement models of Strong *et al.*⁸ and Stecker^{9,10}).

We now consider a model in which particles diffuse from the centre of Virgo and are removed after a mean time T determined by the Hubble time or the attenuation time (λ/c), whichever is the shorter.

Calculations have been made for a variety of forms of the dependence of the diffusion coefficient on energy $D(E)$. General arguments dictate that D should be a function of E but the dependence is not known. For galactic cosmic rays the change of slope of the spectrum at $\sim 3 \times 10^{15}$ eV is often interpreted as due to a change of the form of $D(E)$ from $D(E) =$

constant below 3×10^{15} eV to $D(E) \propto E^{0.5}$ above. The latter is our preferred choice for the supercluster.

The flux of cosmic rays at a distance x from a continuously emitting source can be shown to be

$$n(x) = \frac{qcT}{2\pi^2 x^3} w \phi(\sqrt{2w}),$$

where $w = x^2/4DT$ and

$$\phi(y) = \frac{1}{\sqrt{2\pi}} \int_y^\infty \exp\left(-\frac{s^2}{2}\right) ds \quad (1)$$

where q is the rate of emission of particles from the source. It is assumed that D is only a slowly varying function of E . The expression is valid only at energies significantly below E_0 where $D(E_0) = cx/3$; at significantly higher energies straight line propagation occurs and $n(x) = q/4\pi x^2$ (the flux being collimated from the source). For very low energies, where $\phi(y)$ is given by the recurrence formula,

$$n(x) \rightarrow \frac{cq}{8\pi^{5/2}} \frac{1}{x^2 D} \sqrt{DT} \exp\left(-\frac{x^2}{4DT}\right) \quad (2)$$

Figure 1 shows $n(x)$ for two forms of $D(E)$. The curves are normalised at 10^{19} eV. Above 10^{20} eV there is increasing inaccuracy as straight line propagation is approached and our estimate is only approximate. The forms chosen for $D(E)$ were $D(E) \propto E^{1/2}$ and $D(E) \propto E$ —the galactic situation seems to require the former and we regard this as the preferred expectation for supercluster propagation, at least above 10^{18} eV.

Figure 2 shows the prediction for $D(E) \propto E^{1/2}$ normalised to the experimental results at 10^{19} eV. The required production spectrum is

$$q(E) = 7.4 \times 10^{24} \left(\frac{E(\text{GeV})}{10^{10}} \right)^{-2.44} \text{ s}^{-1} \text{ GeV}^{-1} \quad (3)$$

and the diffusion coefficient is

$$D(E) = 5 \times 10^{33} \left(\frac{E(\text{GeV})}{10^{10}} \right)^{1/2} \text{ cm}^2 \text{ s}^{-1} \quad (4)$$

There is quite good agreement between prediction and observation, at least above about 5×10^{17} eV (in this model, particles of lower energy would come from galactic sources).

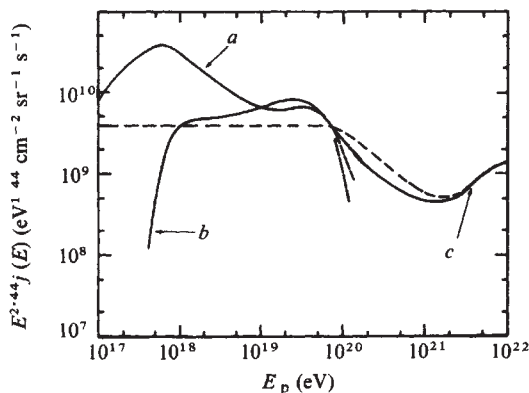


Fig. 1 Spectra of protons expected from the centre of Virgo. Predictions for alternative choices of $D(E)$ are indicated. Chain lines above 10^{20} eV are predictions for the diffusion model in this region; there must be a transition to the situation for straight line propagation (c), which must be reached above about 2×10^{21} eV, and the full line represents an approximate estimate. If there were no extragalactic magnetic field (--- $B_{EG} = 0$) the contribution from Virgo alone would follow the dashed line indicated. The drop above 10^{20} eV is due to interactions with the 2.7 K radiation. An energy-independent $D(E)$ would not give the required upturn in the spectrum. a, $D = 5 \times 10^{33} \sqrt{E(\text{GeV})/10^{10}} \text{ cm}^2 \text{ s}^{-1}$. b, $D = 5 \times 10^{33} (E(\text{GeV})/10^{10}) \text{ cm}^2 \text{ s}^{-1}$.

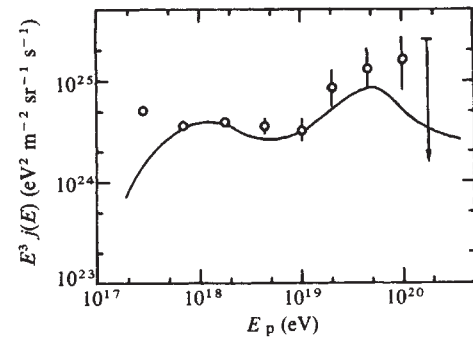


Fig. 2 Spectra of extragalactic protons (from Virgo) predicted using the form of $D(E) \propto E^{1/2}$ given in Fig. 1. The experimental intensities come from a summary of all the experimental measurements⁴⁻⁶.

The numerical values for the parameters can be examined to see if they are reasonable. $D(E)$ gives $\lambda \approx 150$ kpc at $E = 10^{19}$ eV and this is of the expected order of magnitude. The production spectrum, however, gives a high output; extrapolating $q(E)$ back to 1 GeV gives a total rate of energy production of $\sim 7 \times 10^{46} \text{ erg s}^{-1}$, compared with $\sim 6 \times 10^{40} \text{ erg s}^{-1}$ for the Galaxy. This ratio of 10^6 is much bigger than the ratio of 500 or so which pertains at radio wavelengths¹¹. A further difference is that the spectral slope ($\gamma = 2.44$) is less than that associated with galactic particles below 10^{15} eV. However, a number of points can be made in support of the argument: (1) The exponent of the production spectrum for galactic particles is probably significantly smaller than the measured value of 2.6 (and nearer 'our' 2.44) because of the likelihood of the cosmic ray lifetime falling with increasing energy. (2) Other galaxies near Virgo may also be contributing. More important, with the likelihood of a different acceleration mode for M87, the exponent could be different at very low energies and the total energy produced consequently much smaller.

An indication that the yield of particles from the M87 region as a whole is high is provided by observations of the diffuse X-ray flux from this part of the supercluster. Forman *et al.*¹² quote an emissivity of about $10^{43} \text{ erg s}^{-1}$ for this diffuse component, to be compared with our own estimate of $10^{38} \text{ erg s}^{-1}$ for the Galaxy, that is a ratio 10^5 , a result only a factor 10 different from that required on our model for cosmic rays.

Finally some remarks can be made about the expected anisotropy. The magnitude of the anisotropy for particles diffusing from a source in the x direction is $\delta = (3D(E)/CN(x)) \times dN(x)/dx$ and this has been evaluated for energies above 10^{18} eV where, on the present model, the particles are largely extragalactic. The values predicted are $\delta = 1\%$, 4% and 20% for primary energies of 10^{18} , 10^{19} and 10^{20} eV, respectively. Measured anisotropies to date are imprecise, and firm evidence for finite anisotropies is absent above 10^{18} eV; however, there seems to be a trend in phases with increasing energy¹³, not inconsistent with an approach to the Virgo direction above 10^{20} eV. The proof of the present model will clearly rest on the observation of such a collimation of arrival directions at these ultra-high energies.

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An evolution free test for non-zero cosmological constant

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The cosmological constant has recently been questioned because of difficulties in fitting the standard $\Lambda = 0$ cosmological models to observational data^{1,2}. We propose here a cosmological test that is a sensitive estimator of Λ . This test is unusual in that it involves no correction for evolutionary effects. We present here the idealised conception of the method, and hint at the statistical problem that its realisation entails.

Consider a collection of test objects emitting radiation containing spectral lines (so that redshifts may be determined), which are distributed on the surface of a sphere. (Any spherically symmetric, bounded distribution will do; this idealisation is for convenience only.) Let the sphere expand with the local Hubble flow. When observed at a great distance the redshifts of these objects will be confined to some narrow interval ($z - \Delta z, z + \Delta z$), and their distribution on the sky will be confined in some circle of angular radius $\Delta\theta$. Our task is to evaluate $\Delta z/(z\Delta\theta)$.

We discuss only the homogeneous, isotropic cosmologies described by the Robertson-Walker metric³,

$$ds^2 = -dt^2 + R^2(t) \left\{ \frac{dr^2}{1 - kr^2} + r^2(d\theta^2 + \sin^2\theta d\phi^2) \right\} \quad (1)$$

where the dynamical evolution of the Universe is expressed by the equation

$$\dot{R}^2 = R^{-1} \left\{ \frac{\Lambda R^3}{3} - kR + \frac{8\pi}{3} \rho_0 R_0^3 \right\} \quad (2)$$

All symbols have their usual meanings. It is easy to evaluate $\Delta z/(z\Delta\theta)$ by tracing the null geodesics of this geometry. We do this and express the answer in terms of q_0 and Ω_0 by using⁴,

$$\begin{aligned} \rho_0 &= \left(\frac{3}{8\pi} \right) H_0^2 \Omega_0 \\ \frac{k}{R_0^2} &= H_0^2 \left(\frac{3}{2} \Omega_0 - q_0 - 1 \right) \\ \Lambda &= 3H_0^2 \left(\frac{1}{2} \Omega_0 - q_0 \right) \end{aligned} \quad (3)$$

from which we obtain

$$\begin{aligned} \frac{\Delta z}{z\Delta\theta} &= \frac{\left\{ \frac{1}{2} \Omega_0 - q_0 - \left(\frac{3}{2} \Omega_0 - q_0 - 1 \right) (1+z)^2 + \Omega_0 (1+z)^3 \right\}^{1/2}}{z \left[\frac{1}{2} \Omega_0 - q_0 - 1 \right]^{1/2}} \\ &\times \sum_k \left(\left[\frac{1}{2} \Omega_0 - q_0 - 1 \right]^{1/2} \int_1^{1+z} \frac{dy}{\left\{ \frac{1}{2} \Omega_0 - q_0 - \left(\frac{3}{2} \Omega_0 - q_0 - 1 \right) y^2 + \Omega_0 y^3 \right\}^{1/2}} \right) \end{aligned} \quad (4)$$

where

$$\sum_{+1} (x) = \sin x, \quad \sum_{-1} (x) = \sinh x \quad (5)$$

In the case $k = 0$,

$$\frac{\Delta z}{z\Delta\theta} = z^{-1} \{1 - \Omega_0 + \Omega_0(1+z)^3\}^{1/2} \int_1^{1+z} dy \{1 - \Omega_0 + \Omega_0 y^3\}^{-1/2} \quad (6)$$

For the 'conventional' cosmologies where $\Lambda = 0$ there is the simple expression,

$$\frac{\Delta z}{z\Delta\theta} = \frac{(1+2q_0z)^{1/2}}{q_0^2 z} \{q_0 z + (q_0 - 1)((1+2q_0z)^{1/2} - 1)\} \quad (7)$$

Numerical evaluation of equation (7) shows that $\Delta z/(z\Delta\theta)$ is not a powerful estimator of q_0 in the $\Lambda = 0$ case—there is only 11% variation of $\Delta z/(z\Delta\theta)$ between $q_0 = 0$ and $q_0 = 1$ at $z = 2$. However, the general expressions (4) and (6) show great variations of $\Delta z/(z\Delta\theta)$ with the parameters. This is shown in Fig. 1.

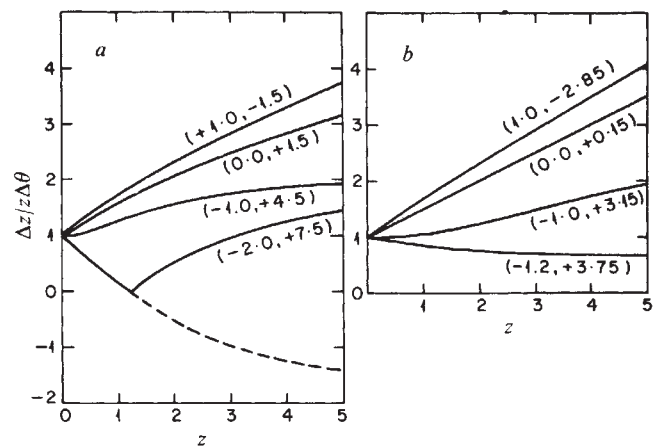


Fig. 1 The variation of $\Delta z/(z\Delta\theta)$ with z is plotted for cosmologies where $a, \Omega_0 = 1.0$; $b, \Omega_0 = 0.1$. Each curve is labelled by $(q_0, \Lambda H_0^{-2})$ where $\Lambda H_0^{-2} = 1.5 \Omega_0 - 3 q_0$. The drastic variations possible in $\Delta z/(z\Delta\theta)$ can be seen even at small z in the event that Λ is significant. The zeros of the lowest curve occur because of antipoles in the null geodesics. The dashed line gives the formal negative result of equation (4), describing apparent reflection of $\Delta\theta$ at the antipoles. The solid curve is the observable result. All of these models have initial singularities.

(Note that with $\Omega_0 = 0.1$, $q_0 < -1.28$ means there was no initial singularity.)

The greatest advantage of this method is its independence of the evolutionary corrections which plague most other tests (the proposal to compare X-ray core radii with microwave diminution measurements is a promising exception^{5,6}). There is the problem, however, that the natural 'test objects' are galaxies in clusters, which have large peculiar velocities. It will be necessary to separate statistically the peculiar and Hubble velocities in a large number of clusters of different angular extent, but at approximately the same mean redshift. This can be done (in principle) because the Hubble velocities scale $\propto \Delta\theta$, whereas the peculiar velocities will have a very different dependence on $\Delta\theta$.

The analysis will require that there be some clustering which is not gravitationally relaxed. Candidate structures which have been observed at low redshifts might be superclusters⁷, or long range correlations around rich clusters⁸. These structures show density enhancements over scales up to $\sim 20 h^{-1} \text{ Mpc}$ (where the Hubble constant h is in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$). The galaxy two-point correlation function could be measured in z and θ . If the observed correlations have the same shape in z and θ one may be confident that pollution of the Δz by peculiar motions is unimportant. (This is true because, in general, gravitational perturbations produce peculiar velocities with the scaling behaviour $\Delta z \propto \Delta\theta^{1/2}$. This will produce a very different

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shape in the correlation function than the linear relation being searched for.) By matching the amplitudes one constructs a measured $\Delta z/(z \Delta \theta)$ which can be used to estimate ΔH_0^{-2} .

It is reasonable to suppose that substantial clustering may occur without the accompanying peculiar velocities. Origins for clustered galaxies have been proposed where, because of dissipation, the peculiar velocities have been damped out. An example is the 'pancake' model⁹ where galaxies form on caustics of the gas motion at redshift ≥ 10 .

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Helium gas-bubble superlattice in copper and nickel

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The microstructural changes occurring in metals during ion bombardment with helium or hydrogen isotopes have important implications in several areas of technology¹ such as in the deterioration of the accelerator targets suggested for use as intense neutron sources in cancer therapy²; and in the plasma-wall interactions which could strongly influence the operation of future thermonuclear fusion reactors (see ref. 3). Research has shown that helium ion irradiation of Mo^{4,5}, Cu, Ni and stainless steel¹ to high doses can result in a superlattice of small (~ 2 nm diameter) helium bubbles in very high concentration ($\sim 10^{25} \text{ m}^{-3}$) even at temperatures up to $0.35 T_m$ (refs 1, 4). The mechanisms involved in the bubble growth⁶ and in the superlattice formation have been discussed but the role of displacement damage collisions on bubble growth has received little study. In the present work, specimens containing a superlattice of helium bubbles formed during prior helium ion irradiation have been irradiated with 1 MeV electrons to create a specific collision regime. This was found to induce bubble growth and changes in superlattice parameters, in both copper and nickel.

An annealed copper target was irradiated in bulk at 300 K with 30-keV helium ions at normal incidence and an electron-transparent specimen containing the bubble layer was prepared. The bubbles lay predominantly on a superlattice having an f.c.c. structure aligned with the copper matrix (see Figs 1a, 3a and ref. 1). The calculated value of the mean projected range was 97 nm with the maximum damage estimated to be ~ 23 dpa (displacements per atom) at depth ~ 80 nm. The subsequent 1 MeV electron irradiation at 300 K was carried out on the Harwell AEI HVEM. The damage dose over an irradiated area $\sim 2 \mu\text{m}$ in diameter was calculated to be 15 dpa and was accumulated in 1 h at a rate similar to that occurring during the ion irradiation at the depth of maximum damage. High resolution microscopy was performed in a Philips EM301 100 keV electron microscope. Electron micrographs taken on, and outside, the 1 MeV electron irradiated area are shown in Fig. 1. For copper, bubble size distributions are shown in Fig. 2, and selected-area diffraction patterns in Fig. 3. It can be seen that following electron irradiation,

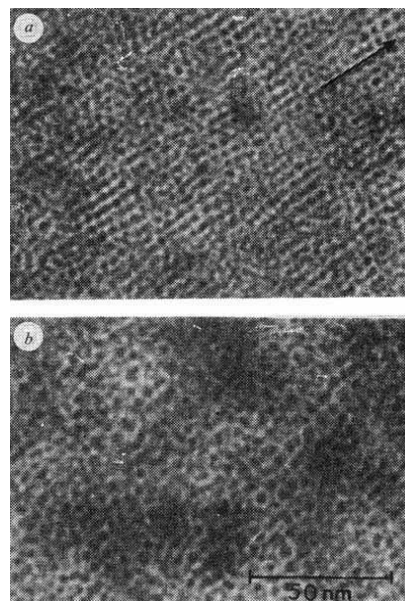


Fig. 1 *a* Direct transmission electron micrograph of helium gas bubbles in copper following helium-ion irradiation to a level of $\sim 4 \times 10^{17}$ 30 keV He ions cm^{-2} at 300 K. The 100 keV electron beam is along [110] in the matrix and is normal to the plane of the bubble-containing layer. The direction of the trace of one set of (111) matrix planes in the (110) plane is indicated. *b*, Direct transmission electron micrograph of the gas bubbles in the same specimen as *a* but in an area, of diameter $\sim 2 \mu\text{m}$, which has been irradiated at 300 K with 1 MeV electrons to increase the damage dose by ~ 15 dpa without the deposition of further helium.

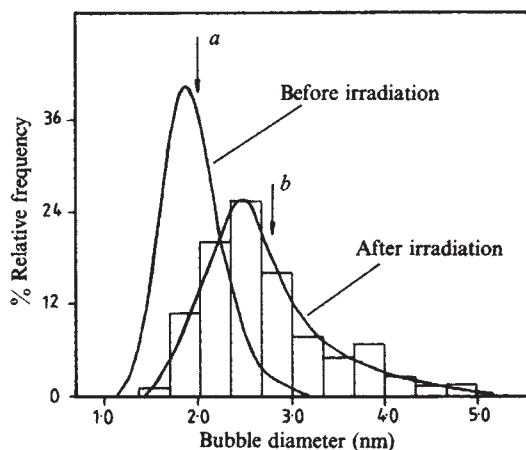


Fig. 2 Bubble size distributions corresponding to Fig. 1a and b. The average bubble diameters (based on average bubble volumes) indicated by the arrows are ~ 2.0 nm and ~ 2.8 nm respectively. It is evident that the 1 MeV electron irradiation has caused a coarsening of the bubble structure.

tion, there is an increase in the average bubble size. A similar result was obtained for nickel in the same conditions. At 300 K the thermal mobility of single vacancies is low in copper and negligible in nickel. The change in the electron diffraction pattern confirms that there has been a genuine change in the bubble structure.

The increase in the bubble lattice constant in copper (from ~ 7.0 nm to ~ 9.0 nm) suggests that bubbles may have moved apart under electron irradiation. Between the domains of good order evident in the bubble structure there are regions which seem less well developed¹. It is possible that local lattice expansions are accommodated by a movement of bubbles outward from the domains into these regions.

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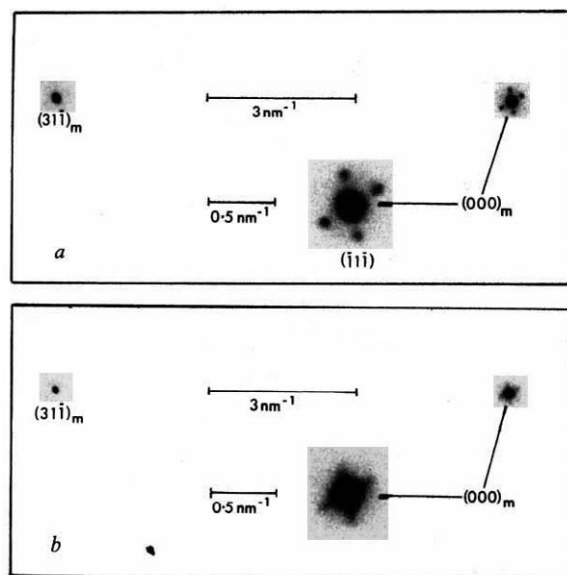


Fig. 3 A section of the $[110]$ selected-area electron diffraction pattern from the copper specimen of Fig. 1. *a* and *b* are respectively outside and on the 1 MeV electron irradiated area. The electron energy is 100 keV. Diffraction from the bubble array causes the splitting that can be seen around the matrix reflections. The superlattice diffraction pattern centred on the (000) transmitted beam is shown enlarged as an inset in each case. Spots which were evident around the (000) reflection in the original negative of *b* have not survived reproduction here. Values for the average spacing between (111) bubble planes, as deduced from the separations of the (111) superlattice spots in the original negatives are ~ 4.0 nm for *a*, and ~ 5.2 nm for *b*. (Quantitative results were not obtained for nickel because the patterns were affected by the magnetic nature of the specimen.) The radial streaking evident in *b* shows that in this case there are interplanar spacings ranging from the quoted value through to higher values.

For irradiation at low temperature Evans⁶ has suggested that the most likely growth mechanism is one in which overpressurised bubbles punch out interstitial dislocation loops. Assuming that this is the mechanism operating and that no significant long-range movement of the helium takes place during implantation, an estimate can be made of the helium content of the visible bubbles before the electron irradiation (P.B.J. and B. Newport, unpublished). For depths of ~ 100 nm only $\sim 15\%$ of the helium implanted seems to be in the bubbles. Although the uncertainties involved are large the general conclusion is that an appreciable fraction of the helium is trapped in the matrix probably in the form of helium-vacancy complexes⁷. The estimated density for helium in bubbles ($\sim 10^{23}$ atoms cm^{-3}) exceeds, by a factor ~ 3 , the average value calculated for helium in the matrix at the same depth. This suggests that some helium atoms have moved to the bubbles from their initial place of implantation in the matrix during the course of the helium irradiation. There is no obvious increase in the bubble size once irradiation is stopped. The continued growth during electron irradiation may be in response to bubbles gaining further helium, and perhaps vacancies, displaced from the matrix as a result of electron-atom collisions. It should be emphasised, however, that there are other possibilities which cannot be ruled out.

That collision or displacement events alone can give rise to bubble growth is an important observation in relation to developing an adequate understanding not only of bubble growth during inert gas or proton irradiation but also of synergistic effects when different types of radiation are present, for example in a fusion reactor where the first-wall surface is expected to be bombarded by a variety of ions and particles of widely varying energy³. The results presented here could be

explained by several different mechanisms acting either individually or in concert. Amongst these are: (1) processes involving the detrapping of helium from helium-vacancy complexes; (2) the radiation assisted migration of helium; (3) the dynamic re-solution of helium from existing bubbles and its redistribution; and (4) the athermal radiation stimulated migration of vacancies to the bubbles⁸. These and other mechanisms will need to be investigated in further experiments to elucidate their roles more precisely.

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Identification of clear taenite in meteorites as ordered FeNi

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In the Fe–Ni system, the face-centred cubic (f.c.c.) phase (called taenite in meteorites) is stable for all compositions above 1,185 K. Below this temperature, the body-centred cubic phase (kamacite) is stable and has a maximum Ni concentration of about 7% around 720 K (ref. 1). Metal in meteorites is commonly found to contain kamacite with 4–7% Ni and taenite with 20–45% Ni. Taenite grains have M-shaped Ni profiles which formed on slow cooling during diffusion-controlled growth of kamacite and preserve a record of the cooling rates. Another Fe–Ni phase with 49–57% Ni was found in ordinary chondrites by Taylor and Heymann² and identified as a variety of taenite. They called it clear taenite to distinguish it from the zoned variety, which develops cloudy borders on etching with acid, and proposed that it nucleated at 660–610 K on the edge of isolated kamacite grains. We report here studies of meteoritic metal with reflected-light microscopy and electron-probe analysis, in which we discovered that clear taenite is optically anisotropic and we have found it in all meteorite groups. We use the term clear taenite as originally defined to include only alloys with ~ 48 –57% Ni, not those with ~ 25 –30% Ni, which Lin *et al.*³ called clear taenite II. Except for cloudy taenite which contains ~ 30 –40% Ni and looks cloudy because it is a submicroscopic two-phase mixture, taenite remains clear on etching. We conclude that clear taenite is the ordered, tetragonal FeNi phase found by Albertsen *et al.*^{4–6} in two iron meteorites using Mössbauer and X-ray techniques, and that it formed from taenite, not kamacite. This tetragonal phase has been synthesised by irradiating FeNi alloys with neutrons or electrons below 593 K (refs 7, 8).

Optical anisotropy in clear taenite is best observed in well polished sections using oil-immersion objectives and a powerful

Table 1 Range and mean composition in wt % of 25 clear taenite grains in eight chondrites and mesosiderites determined by electron-probe analysis

	Range	Mean
Ni	47–57	51
Fe	44–53	49
Cu	0.11–0.38	0.24
Co	0.01–0.8	0.03
P	<0.01	<0.01

source of polarised light. Contrast can often be improved by slightly uncrossing the polars. Clear taenite shows a characteristic pattern of irregular grains (Fig. 1) in which subdomains are usually visible. These subdomains show flame-like or more intricate arrangements and are probably magnetic domains judging from their similarity to those seen in cohenite (Fe_3C) in the same conditions. From the descriptions of Gooley *et al.*⁹ it is evident that the mineral in which they found optical anisotropy was in fact Taylor and Heymann's clear taenite. Careful observations of a few large areas of clear taenite reveal three distinct orientations of grains, consistent with the symmetry change from cubic to tetragonal which occurs on ordering in FeNi. Because etching may cause apparent anisotropy, we used only unetched sections to characterise this mineral. In such sections, clear taenite is virtually identical in colour to the zoned taenite which is adjacent, although their interface may sometimes be observed without crossed polars.

In addition to the 10–60- μm diameter grains which Taylor and Heymann² identified, clear taenite occurs in sharply defined rims on normal zoned grains. These rims, which are 1–5 μm wide, contain 48–57% Ni and under crossed polars show an irregular grain structure which is characteristic of clear taenite. On etching it can be seen that the anisotropic taenite rim

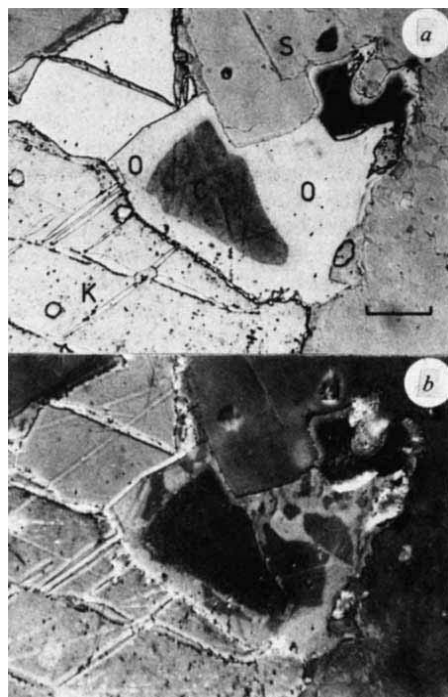


Fig. 1 Reflected light photomicrograph of metal grain in Mezö-Madaras, an L3 meteorite, etched with acid to reveal structure; *a*, polarised light; *b*, crossed polars. In *a* cloudy taenite (C) is surrounded by a white rim of clear taenite (O). Crossed polars show that clear taenite is optically anisotropic and reveal an irregular grain structure. Clear taenite is surrounded by kamacite (K) and silicate (S). (Polished section is carbon coated; this enhances contrast between grains in clear taenite by reducing the visibility of the subdomains.) Scale bar, 20 μm .

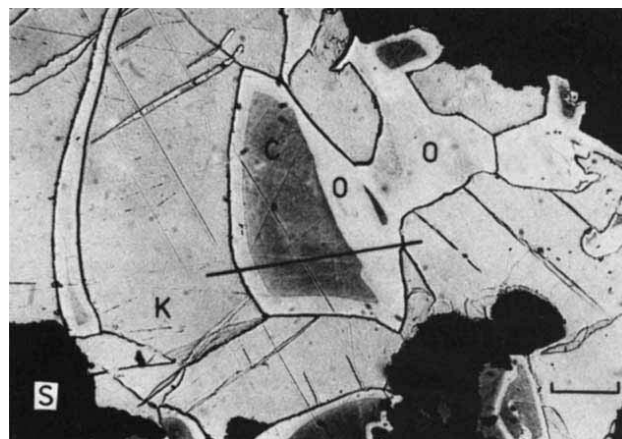


Fig. 2 Reflected light micrograph of metal grain in Mezö-Madaras. The line across the cloudy taenite grain (C) which is rimmed by ordered clear taenite (O) marks the location of the compositional profile shown in Fig. 3. Kamacite, K; silicate, S; scale bar, 20 μm .

corresponds precisely to the zone of clear taenite between kamacite and cloudy taenite (Fig. 1). Electron probe traverses zoned taenite grains with broad rims in over 10 mesosiderites and chondrites (Figs 2 and 3) show a relatively homogeneous rim and a discontinuity at the interface between clear and cloudy taenite like that found in Estherville¹⁰. These features in the M-profile were not resolved by other workers (for example, refs 11, 12) because they did not analyse small favourably located grains with broad rims.

We have found optical anisotropy in clear taenite from over 40 meteorites including H, L, LL and CO3 chondrites, diogenites, iron meteorites, mesosiderites and pallasites and conclude that all slowly cooled meteorites with zoned taenite contain some clear, anisotropic taenite also. Table 1 summarises the compositions of clear taenite found by electron-probe analysis of eight meteorites. With the exception of reheated meteorites and heat-affected surfaces we have not found normal isotropic taenite with compositions in the range 46–60% Ni.

The intimate association of clear taenite with zoned taenite and its growth on grain boundaries within polycrystalline taenite show that few clear taenite grains could have formed in isolated kamacite grains as Taylor and Heymann² suggested. Instead we propose that large clear taenite, which is found in ordinary chondrites and mesosiderites, formed by the inward growth of clear taenite rims at low temperatures (<700 K). In the mesosiderites Estherville and Emery and the L chondrite Mezö-Madaras, we have seen complete sequences of structures ranging from large clear taenite grains enclosing micrometre-sized blobs of cloudy taenite to large-zoned grains with micrometre-wide rims of clear taenite enclosing cloudy taenite. In most chondrites the intermediate stages in this sequence are less common and there seem to be two distinct populations of grains. Diffusion of Ni through up to 30 μm of clear taenite, which is required in our mechanism, may be facilitated by the numerous grain boundaries.

Even in pallasites and slowly cooled iron meteorites, massive clear taenite grains are absent and the clear rims on zoned grains are relatively narrow compared with those in chondrites. This may be a result of relatively high metallic concentrations of P, which reduce the equilibrium concentration of Ni in taenite¹³. Taenite with about 50% Ni has also been reported in shocked sulphides in irons¹⁴. Other processes must be responsible for its formation there.

The anisotropic FeNi mineral which we have described is undoubtedly the ordered phase reported by Albertsen *et al.*^{5,6} in two iron meteorites. They identified it by the similarity of its Mössbauer spectrum to that of tetragonal FeNi synthesised with neutron irradiation^{7,8} and the presence of X-ray reflections

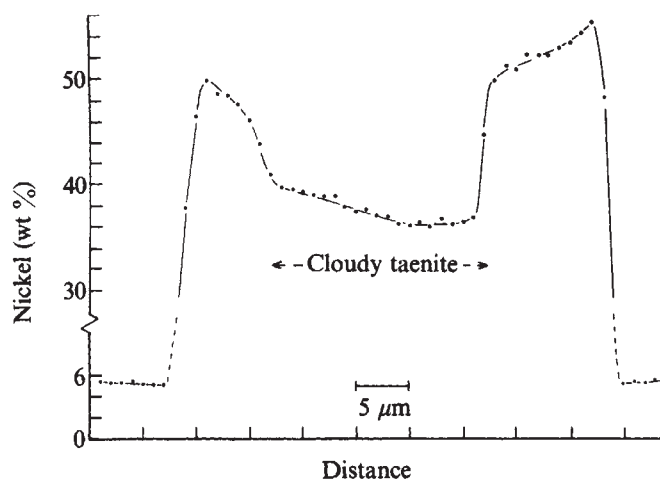


Fig. 3 Electron probe Ni profile across composite taenite grain shown in Fig. 2. The Ni profile is not smooth as others have found; instead there are discontinuities between the clear taenite rim which has 46–55% Ni and the cloudy taenite which has less than 40% Ni.

which violated the f.c.c. lattice of taenite. The only possible ordering arrangement which satisfied their data was the tetragonal L10 structure (AuCu), which can be derived from taenite by ordering Fe and Ni atoms on alternate (002) planes. From etching experiments on zoned taenite lamellae, Albertsen *et al.*⁶ deduced that FeNi was concentrated in the outer rims of these lamellae. Our observations and probe analyses of anisotropic rims are entirely consistent with their work, and taken together they characterise clear taenite containing 48–57% Ni as a new mineral.

Ordered FeNi areas several micrometres in size have also been reported^{15,16} in another iron meteorite, Santa Catharina, which contains 34% Ni (ref. 14). Nickel could not have been concentrated in this iron by growth of kamacite, which is virtually absent; instead, local oxidation of Fe has been suggested as a mechanism by Bowles *et al.*¹⁵ but refuted by Danon *et al.*¹⁶. Although the meteorites in which we observed clear taenite are not all entirely unoxidised, the areas which we studied were free from visible oxidation.

As the phases in cloudy taenite can only be resolved by electron microscopy^{3,10} we cannot confirm that cloudy taenite also contains ordered FeNi (refs 4, 5). It is plausible that exsolution in the cloudy zone is triggered by ordering of FeNi, possibly through spinodal decomposition. Electron microscopy of Santa Catharina by Jago¹⁷ did show two submicrometre taenite phases in a structure which seemed to be a precursor to cloudy taenite. However he detected extra reflections consistent with ordering in taenite (and kamacite) only after the second taenite had disappeared on heating at 200 °C.

Estimates of cooling rates^{11,12} ignore the existence of ordered FeNi rims and the two-phase nature of cloudy taenite. Although this is unlikely to cause large errors, a quantitative investigation of these effects is needed. The abundances of clear and cloudy taenites provide a useful visual estimate of the cooling rate as there are rough inverse correlations between abundances and cooling rates.

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On the total CO₂-titration alkalinity-oxygen system in the Pacific Ocean

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Decomposition of organic matter changes the concentrations of carbon, nitrogen, phosphorus, oxygen and titration alkalinity (TA) in the ratio 106:16:1:138:–17 (refs 1–3), so the combined effect of decomposing x mol of CaCO₃ and y mol of organic matter in 1 kg of seawater on the preformed total CO₂ (ΣCO_2), preformed TA (TA⁰), biogenerated ΣCO_2 [$\Delta\Sigma\text{CO}_2(\text{biol})$] and the apparent oxygen utilisation (AOU) can be represented as⁴

$$\Delta\Sigma\text{CO}_2 = \Sigma\text{CO}_2(\text{measured}) - \Sigma\text{CO}_2^0 = x + 106y$$

$$\Delta\text{TA} = \text{TA}(\text{measured}) - \text{TA}^0 = 2x - 17y$$

$$\Delta\Sigma\text{CO}_2(\text{biol}) = 106y; \quad \text{AOU} = 138y$$

Eliminating x and y from the above equations yields

$$\Delta\Sigma\text{CO}_2(\text{biol}) = 0.768 \text{ AOU} \quad (1)$$

$$\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA} = 0.83 \text{ AOU} \quad (2)$$

Several early workers^{5–8} have successfully correlated $\Delta\Sigma\text{CO}_2(\text{biol})$ to AOU with slopes close to the Redfield, Ketchum and Richards (RKR) model¹. However, the methods used often have not satisfactorily taken into account the variation of the preformed values for ΣCO_2 and TA with sample depth or have mistakenly taken $\Delta\Sigma\text{CO}_2(\text{biol})$ to be equal to $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$. The inconsistency between the correlations above and below the thermocline is also not well explained. A modified computational scheme^{5,8} has recently been developed for calculating the ΣCO_2 -TA-oxygen correlation with the depth dependent variations of ΣCO_2^0 and TA⁰ accounted for. The method of calculating $\Delta\Sigma\text{CO}_2$ is the same as that described in ref. 4 but further useful information has been obtained by plotting $\Delta\Sigma\text{CO}_2$ against AOU, rather than depth. The results in the Pacific Ocean, presented here, suggest a linear correlation between the bio-generated CO₂ and AOU with a slope of 0.722 ± 0.05 , in good agreement with that predicted from the RKR model. The deviation of the data from this linear correlation for shallow water can largely be explained by the influences of human induced CO₂.

The values for ΣCO_2^0 and TA⁰ are needed in order to calculate $\Delta\Sigma\text{CO}_2$ and ΔTA . It has been shown^{8,9} that the surface values of ΣCO_2 and TA show a linear correlation with temperature. Using more recent data (Geochemical Ocean Section Study, Atlantic Ocean), Chen and Millero⁴ have confirmed that TA⁰ and ΣCO_2^0 show a linear temperature dependence on the constant salinity basis, but only at the lower temperatures. We have, therefore, normalised the values for ΣCO_2^0 and TA⁰ in the Pacific Ocean (GEOSECS) to 35‰ salinity using

$$\Sigma\text{CO}_2^0(\text{or TA}^0) = \frac{\Sigma\text{CO}_2^0(\text{measured}) [\text{or TA}^0(\text{measured})] \times 35.000}{S(\text{‰, measured})}$$

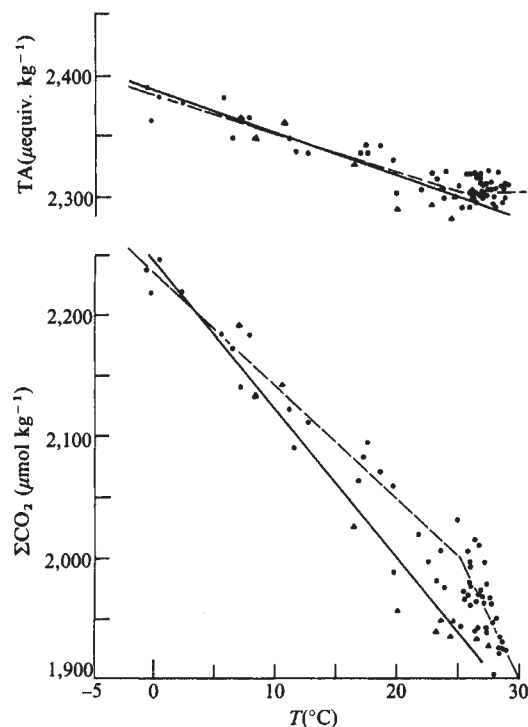


Fig. 1 The surface TA and ΣCO_2 values, normalised to 35‰ salinity, at various temperatures. The triangles and the solid line represent the data in the Northwest Pacific. The circles and the dashed line represent the data elsewhere in the Pacific Ocean.

These normalised values also correlate with the potential temperature in the Pacific Ocean although they seem to fall in two groups (Fig. 1). Stations in the north-west region (shaded area on Fig. 2) seem to have different values for ΣCO_2 and TA^0 than the rest of the Pacific Ocean. As a result, the following equations are used to represent ΣCO_2 and TA^0 in the north-west region (triangles, represented by solid lines on Fig. 1):

$$\begin{aligned}\Sigma\text{CO}_2^0(\mu\text{mol kg}^{-1}) &= 2,242 - 12.08\theta (\pm 18) \\ \text{TA}^0(\mu\text{equiv kg}^{-1}) &= 2,384 - 3.36\theta (\pm 11)\end{aligned}\quad (3)$$

Whereas, the following equations are used to represent the values elsewhere in the Pacific (circles, represented by dashed lines on Fig. 1):

$$\begin{aligned}\Sigma\text{CO}_2^0(\mu\text{mol kg}^{-1}) &= 2,236 - 9.4\theta (\pm 21) \quad \theta \leq 25^\circ\text{C} \\ &= 2,500 - 20\theta (\pm 23) \quad \theta > 25^\circ\text{C}\end{aligned}\quad (4)$$

$$\begin{aligned}\text{TA}^0(\mu\text{equiv kg}^{-1}) &= 2,383 - 3.1\theta (\pm 10) \quad \theta \leq 25^\circ\text{C} \\ &= 2,306 (\pm 10) \quad \theta > 25^\circ\text{C}\end{aligned}\quad (5)$$

The numbers in parentheses give one standard deviation of the least squares fit for the above equations. The salinity and higher order temperatures terms are not statistically significant.

The GEOSECS station GS 347 has been chosen as a demonstration station. The values for $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ calculated from equations (2)–(5) are plotted against AOU in Fig. 3. The dashed line (a) on Fig. 3 has a slope of 0.83, therefore, one might expect the data points to fall on the line if both the computational scheme and the RKR model are correct. This is clearly not the case. Although the sample at 20 m falls near the theoretical line, that at 189 m falls away from it and that at 336 m falls even further away. The samples below 589 m deviate from line a the most but seem to correlate linearly with AOU (line b) with a slope of 0.76 ± 0.05 similar to that predicted by the RKR model.

Two possibilities may explain the phenomenon, which is typical for the stations we examined. First, that the RKR model is invalid in the near-surface layer but becomes valid for deep

waters—this seems unreasonable. Second, there may be a deficiency in our computational scheme—the values of present-day ΣCO_2^0 used to calculate $\Delta\Sigma\text{CO}_2$ might not represent the values of ΣCO_2^0 for waters formed in the past.

Human activities, mainly the burning of fossil fuels and possibly also the cultivation of virgin lands and clearing of forests, have significantly increased the amount of CO_2 in the atmosphere over the last 130 years^{10–18}. Recent work^{4,19–21} has shown that the human-induced CO_2 has penetrated into the oceans. Consequently, the ΣCO_2^0 values calculated from the present-day data probably are higher than the ΣCO_2^0 values for old waters. The values for TA^0 probably do not vary with atmospheric CO_2 and we will assume that the present-day TA^0 values represent the values of TA^0 for water of various ages in our discussion.

Since the values of ΣCO_2^0 for younger waters are closer to the present-day ΣCO_2^0 values (equations (3) and (4)), the computational scheme is 'more correct' for younger waters. This agrees with the data shown on Fig. 3; the shallower, and presumably, younger samples fall closer to the theoretical line. The waters below 589 m deviate from the line by a nearly constant amount which indicates that the ΣCO_2^0 values for these waters deviate from the present day ΣCO_2^0 by approximately a constant amount. In other words, the waters below 589 m probably have not felt the influence of the atmospheric CO_2 variations. Although the ^{14}C and tritium have different source function and equilibration time as compared to CO_2 , their vertical distributions²² seem to substantiate this conclusion, at least qualitatively (Fig. 3). Very little tritium, but large negative values for $\Delta^{14}\text{C}$ have been found below 590 m.

It is generally accepted that protein is utilised more readily than carbohydrate as particulate organic matter is transported from surface to deep waters^{23–26}, consequently, the C/N/P/O ratio changes accordingly. However, our results indicate that since the changes in the masses of nitrogen and phosphorus are small as compared to the changes in the masses of carbon and oxygen, the changes in the C/N, C/P, N/P, N/O and P/O ratios probably do not significantly affect the C/O ratio. The preferential retention of carbon over oxygen in upper waters could indeed change the C/O ratio. However, this process has to be significantly effective everywhere from surface to 589 m deep. No known process and no experimental data support this. As a result, the increase of ΣCO_2 due to the bioregeneration correlates linearly with AOU (line b on Fig. 3) below 589 m with a slope close to the value predicted by the RKR model.

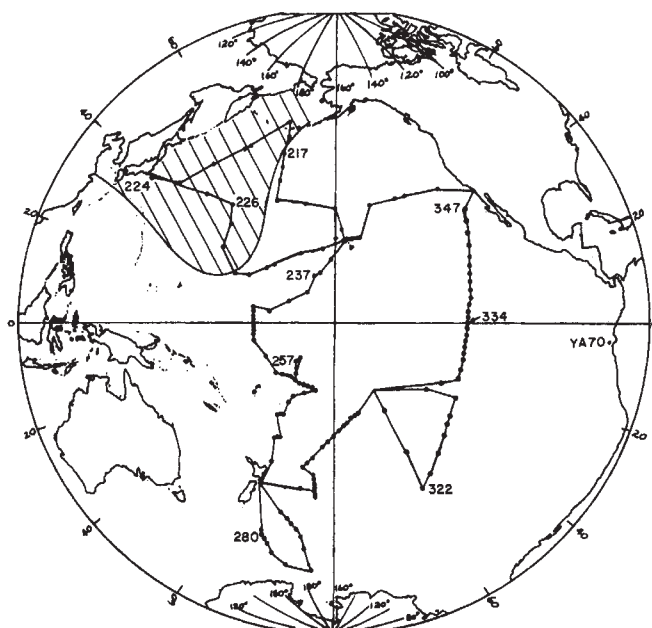


Fig. 2 Station locations in the Pacific Ocean.

The deviation of the data from the predicted values in the upper layer probably is largely caused by the influx of human induced CO_2 . The best linear fit of the values for $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ gives a value of $41 \pm 15 \mu\text{mol kg}^{-1}$ (the distance between O and A) at $\text{AOU} = 0$. The value of $41 \pm 15 \mu\text{mol kg}^{-1}$ probably represents the change in ΣCO_2^0 ($\Delta\Sigma\text{CO}_2^0$) due to the change of CO_2 concentration in the atmosphere. (The uncertainty of $\pm 15 \mu\text{mol kg}^{-1}$ reflects only the random error due to scatter of the observed data and does not include any systematic error.) Integrating $\Delta\Sigma\text{CO}_2^0$ from surface to 589 m gives a total CO_2 increase of 1.2 mol cm^{-2} . As a very crude first approximation, we assumed that the average difference in the concentration of man-made ΣCO_2 was $5 \pm 5 \mu\text{mol kg}^{-1}$ between surface and 589 m over the last 130 yr and arrived at an eddy diffusion coefficient of $3.5 \pm 3.5 \text{ cm}^2 \text{ s}^{-1}$. The value is in reasonable agreement with the findings of Pytkowicz for oxygen²⁷ and Oeschger *et al.* for CO_2 ²⁸.

Data from stations widely scattered in the Pacific Ocean (Fig. 2; GS 217, 224, 226, 237, 257, 280, 322, 334, 347 and YALOC 69 station 70) have also been examined and the results follow the same pattern as GS 347. The values for $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ for these stations have been plotted against AOU in Fig. 4. These values from the deep layers (filled circles; depth varies from below 100 m at YA 70 to below 1200 m at GS 226 and 322) correlate linearly (line *b*) with AOU with an average slope of 0.78 ± 0.05 , which corresponds to a slope of 0.722 between $\Delta\Sigma\text{CO}_2$ (biol) and AOU. The data from the shallower layers (open circles) deviate from this best fit straight line with the near surface values centred around (0, 0) interception. Line *a* has the same slope as line *b* but is drawn through the (0, 0) interception. The vertical distance between a data point and line *a* probably represents the difference between the previous and present ocean surface ΣCO_2 concentrations.

Note that our computational scheme represents only first approximations of the very complicated system in the oceans⁴. Although the decrease of TA^0 and ΣCO_2^0 with increasing temperatures can be qualitatively explained as due to the decrease in the solubility for CaCO_3 and CO_2 , the magnitude of the decrease cannot yet be estimated theoretically. There is also

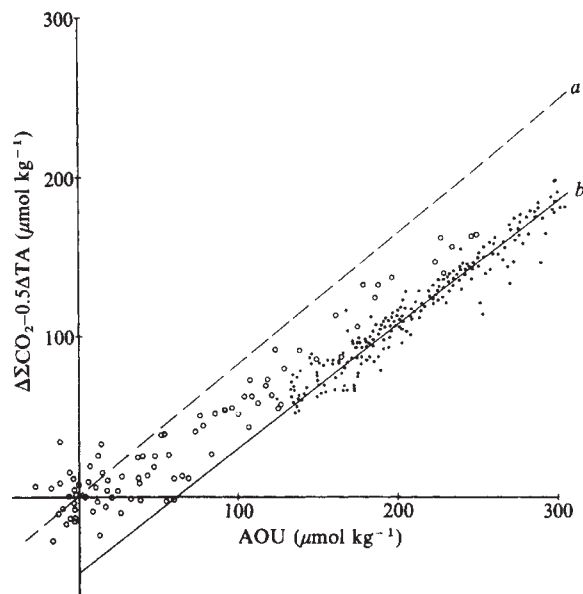


Fig. 4 The relationship between $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ and AOU at 10 stations in the Pacific Ocean. Line *b* is the best fit straight line for the old water samples (filled circles). Line *a* has the same slope as line *b* but is drawn through the (0, 0) interception.

no satisfactory explanation as to why the correlations of TA^0 and ΣCO_2^0 with temperature change slope at higher temperatures. Whether they are caused by the change in the biological activity, or by the change in current flow patterns, or other physical processes such as equatorial upwelling²⁹ deserves further investigation. Moreover, the GEOSECS summer data in the Pacific Ocean have been used in our calculations although the winter TA^0 and ΣCO_2^0 concentrations in the Atlantic and Pacific polar regions where intensive vertical mixing occurs may be different from the Pacific summer data. This summer-winter incompatibility may cause a systematic error in addition to the random error. However, our preliminary estimation indicates that the $\Delta\Sigma\text{CO}_2^0$ value calculated here is unlikely to be systematically too high by more than $15 \mu\text{mol kg}^{-1}$. A maximum value of $26 \mu\text{mol kg}^{-1}$ in $\Delta\Sigma\text{CO}_2^0$ would give an eddy diffusion coefficient of $2.2 \pm 2.2 \text{ cm}^2 \text{ s}^{-1}$, which is in good agreement with the results of Pytkowicz²⁷ and Oeschger *et al.*²⁸. The slope of the $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ against AOU correlation would remain essentially the same, however.

These data were obtained as part of the Pacific GEOSECS Expedition carried out aboard RV Melville of the Scripps Institution of Oceanography during 1973 and 1974. The ship-board work was largely carried out by personnel of the GEOSECS Operations Group under the direction of the late Arnold E. Bainbridge.

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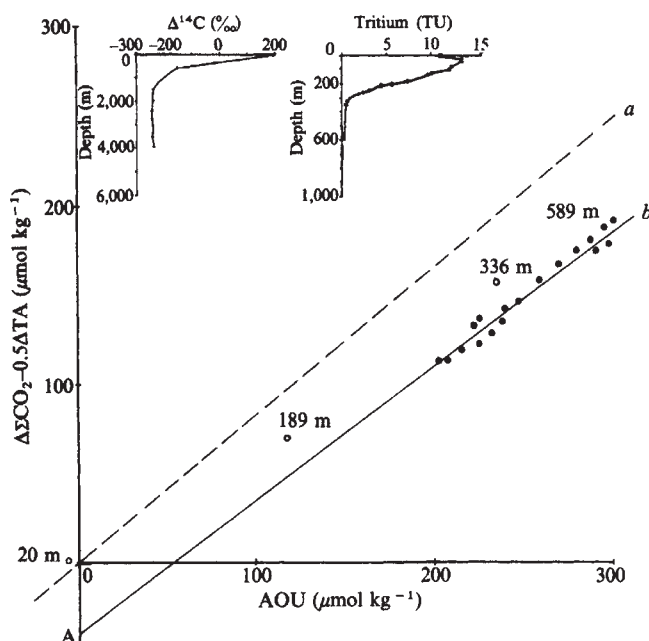


Fig. 3 The relationship between $\Delta\Sigma\text{CO}_2 - 0.5\Delta\text{TA}$ and AOU at GS 347. Open circles denote young waters and filled circles, old waters. Line *a* represents the theoretical correlation predicted from the RKR model. Line *b* is the best-fit correlation for the old water samples. The insert shows the tritium (in tritium units, TU) and $\Delta^{14}\text{C}$ (‰) profiles.

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Primitive lead in deep crustal xenoliths from the Snake River Plain, Idaho

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The petrology and geochemistry of deep crustal rocks are poorly understood due to the inaccessibility of such materials. Most inferences of the lower crust are based on studies of ancient high-grade metamorphic terranes now exposed after a history of deep burial. However, direct sampling of deep crustal rocks is feasible when they are carried to the surface as accidental fragments (or xenoliths) in lava flows. Ferro-basalt to ferro-latitude lavas from Craters of the Moon lava field (COM) in the Snake River Plain, southern Idaho are characterised by variable and high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (0.708–0.712) interpreted as reflecting variable degrees of crustal contamination^{1,2}. Some of these lavas contain xenoliths of crustal derivation (silicic charnockites and pyroxene granulites) that are found nowhere in the vicinity in surface outcrops. I present here initial isotopic and trace element results for a suite of these crustal xenoliths. As part of a detailed Pb isotopic study of the lavas (in preparation), three granulites were analysed to test the contamination hypothesis. The results indicate an affinity between the xenoliths and many exposed granulite-facies metamorphic terranes, and the unusually primitive lead isotopic ratios recorded establish the presence of early Archean crust beneath south-central Idaho.

The crustal xenoliths all contain quartz, plagioclase, Fe–Ti oxides, and hypersthene $\pm$ clinopyroxene $\pm$ alkali feldspar. These mineral assemblages place the rocks in the granulite facies at intermediate crustal depths. Three fresh xenoliths, 15–50 cm in approximate diameter, were sampled by drilling 1.2-cm cores, weighing about 30–50 g, from central portions of the rocks. In one case a core was taken near the xenolith rim to test for contamination from the host lava. The cores were washed in dilute HCl and deionised triple-distilled H₂O, crushed in a WC mill, digested in HF–HClO₄ in Teflon beakers in a low-Pb laboratory. Pb was separated by a Ba(NO₃)₂ precipitation and purified by anodic electrodeposition. Pb isotopic compositions and trace element concentrations were determined on separate dissolutions. Analyses were made on a 30 cm NBS-design mass spectrometer using the Si-gel method and isotopic compositions were corrected for mass fractionation by the double-spiking method³. Blanks run with these samples averaged about 5 ng and are insignificant for the reported analyses. All isotopic ratios are accurate to about 0.15% relative to NBS standard SRM-981. Pb, U, Th, Rb and Sr concentrations were determined by isotope dilution methods and are accurate to within 3% of the amount present. Table 1 shows replicate analyses (full procedural duplicates).

Isotopic compositions of the xenoliths are extremely primitive and are quite distinct from the host and all other analysed COM lavas. Core and rim analyses of xenolith CKI-1 are marginally distinct, but in fact the rim sample is the least radiogenic and seemingly precludes addition of Pb to the xenolith from the host lava. Isotopic ratios for this xenolith are among the least radiogenic values reported from the US. The $^{206}\text{Pb}/^{204}\text{Pb}$ and $^{207}\text{Pb}/^{204}\text{Pb}$ ratios plot near the 2,900 Myr isochron through the Stacey–Kramers growth curve for conformable ore leads⁴ and

Table 1 Isotopic and trace element data for COM granulite xenoliths*

K	Rb	Sr	Pb	U	Th	Rb/Sr	Th/U	μ	$^{87}\text{Sr}/^{86}\text{Sr}$	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$
V-31 Devils Orchard flow (ferro-latitude)												
3.88	97	175	31.1	3.25	11.8	0.554	3.6	6.59	0.7110	17.81	15.60	38.51
										17.82	15.62	38.56
										17.81	15.60	38.52†
SI-1 charnockite												
0.86	6.34	69.8	9.02	0.36	33.2	0.091	92.2	3.17	0.7173	16.08	15.62	58.97
										16.11	15.70	59.26
										16.09	15.64	59.07
70-40 hypersthene granulite												
2.01	22.2	325	22.4	<0.2	6.09	0.068	>30	<0.50	0.7152	14.12	14.84	35.77
										14.09	14.81	35.70
										14.11	14.82	35.73
CKI-1 charnockite (core)												
2.18	30.0	235	15.8	0.061	0.104	0.128	1.6	0.215	0.7271	13.55	14.70	35.00
										13.56	14.73	35.03
										13.56	14.72	35.01
CKI-1 (rim)												
—	—	—	16.3	—	—	—	—	—	—	13.42	14.67	34.65
										13.44	14.70	34.75
										13.43	14.68	34.70

Internal precision (2σ) on isotopic ratios is <0.0003 ($^{87}\text{Sr}/^{86}\text{Sr}$), <0.02 ($^{206}\text{Pb}/^{204}\text{Pb}$), <0.03 ($^{207}\text{Pb}/^{204}\text{Pb}$), and <0.06 ($^{208}\text{Pb}/^{204}\text{Pb}$) for typical analyses.

* All concentrations in p.p.m. except K (%).

† Weighted mean for the replicate analysis, considering analytical errors for the individual ratios.

indicate that CKI-1 represents a terrane at least that old. Other reasonable growth curves (for example, a single-stage growth curve with a $^{238}\text{U}/^{204}\text{Pb}$ value of about 8.5 and Th/U value of about 6) yield model lead isotopic ratios similar to those in CKI-1 at about 2,900 Myr ago. The actual age of the xenolith (or strictly speaking, the age of granulite metamorphism) is probably slightly greater than 2,900 Myr because no correction has been applied to the observed isotopic ratios to account for *in situ* decay of U and Th isotopes. This correction is not large for CKI-1 due to its low U and Th contents. Acid leaching studies are in progress to estimate more closely the initial lead isotopic composition of this rock. The other xenoliths are somewhat more radiogenic than CKI-1 in accordance with their higher U and Th contents. The model age for CKI-1 is supported by a $^{207}\text{Pb}/^{204}\text{Pb}$ – $^{206}\text{Pb}/^{204}\text{Pb}$ secondary isochron age of 2,870 Myr for the pair of xenoliths, CKI-1 and 70–40. However, a secondary isochron age of 3,760 Myr is indicated for the pair, SI-1 and CKI-1; this result probably reflects complex pre-metamorphic evolution of the lead in these two rocks. Further work is required to define the pre-metamorphic history of the COM xenoliths.

All of the xenoliths have high K/Rb ratios (730 to 1,360) and very low U contents and two have low Th contents. These geochemical features are characteristic of high-grade metamorphic terranes⁵. Considering their primitive lead isotopic compositions, it is clear that the low U and Th contents reflect depletions of these elements during an ancient

metamorphic event and cannot have resulted from interaction with the host lavas. Thus it is clear that these few samples record the presence of a deep Archean basement complex at least several hundred kilometres west of other known outcrops in the northern Rocky Mountains. Archean metasedimentary rocks (2,500 Myr) occur south of the Snake River Plain in the Albion Range⁶, but these are of a lower metamorphic grade than the COM xenoliths.

Sr isotropic ratios for the COM xenoliths are high and, with the low Rb/Sr ratios, provide a further indication of the antiquity of the underlying crust. The few available Rb–Sr data¹ are too scattered to provide a whole-rock isochron. If the model lead age is accepted, it is likely that the xenoliths were characterised by varied initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios unless the Rb–Sr systematics have been disturbed.

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Biopedological origin of peatlands in South East Alaska

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Peatlands are the final stage in plant succession on level surfaces in South East Alaska, a region where bogs and forests in the process of turning into bogs occupy nearly as much surface area as do forests proper^{1–3}. Plant succession in this area is in part conditioned by progressive bogging⁴; however, no previous attempts have been made to relate paludification to soil development. We now propose that the formation of peatlands in the Lituya Bay area of South East Alaska is caused by the deterioration of the internal drainage of the soil as an iron-cemented pan develops during the process of podzolisation. This situation provides a clear example of soil-induced bog formation, a process that may be important in other parts of the world.

Along the coast of the Gulf of Alaska, in Glacier Bay National Monument, tectonic uplift along the active Fairweather Fault and eustatic sea-level changes have produced marine terraces (Fig. 1). The wave-planed, seaward-sloping bedrock of the terraces is covered by 2–4 m of beach sands and gravels. The terraces provide a chronosequence of surfaces on which plant communities and soils have developed.

Terrace A, the lowest, is 150 m wide with an average elevation of 9 m above sea level; its minimum age is estimated at 400 yr from the ages of the oldest trees (Fig. 2). Terrace B is approximately 250 m wide with an average elevation of 26 m; its estimated age is $2\text{--}3 \times 10^3$ yr (ref. 5). Terrace C is 1,600 m wide with an average elevation of 80 m, and is estimated to be $6\text{--}8 \times 10^3$ yr old⁵.

We investigated five transects across these terraces near Lituya Bay. For each transect, soils and the vegetation were described, and the ages of the largest trees determined by coring.

We will describe here the results from a representative transect, the Steelhead Transect, 10 km south-east of Lituya Bay (Figs 1, 2).

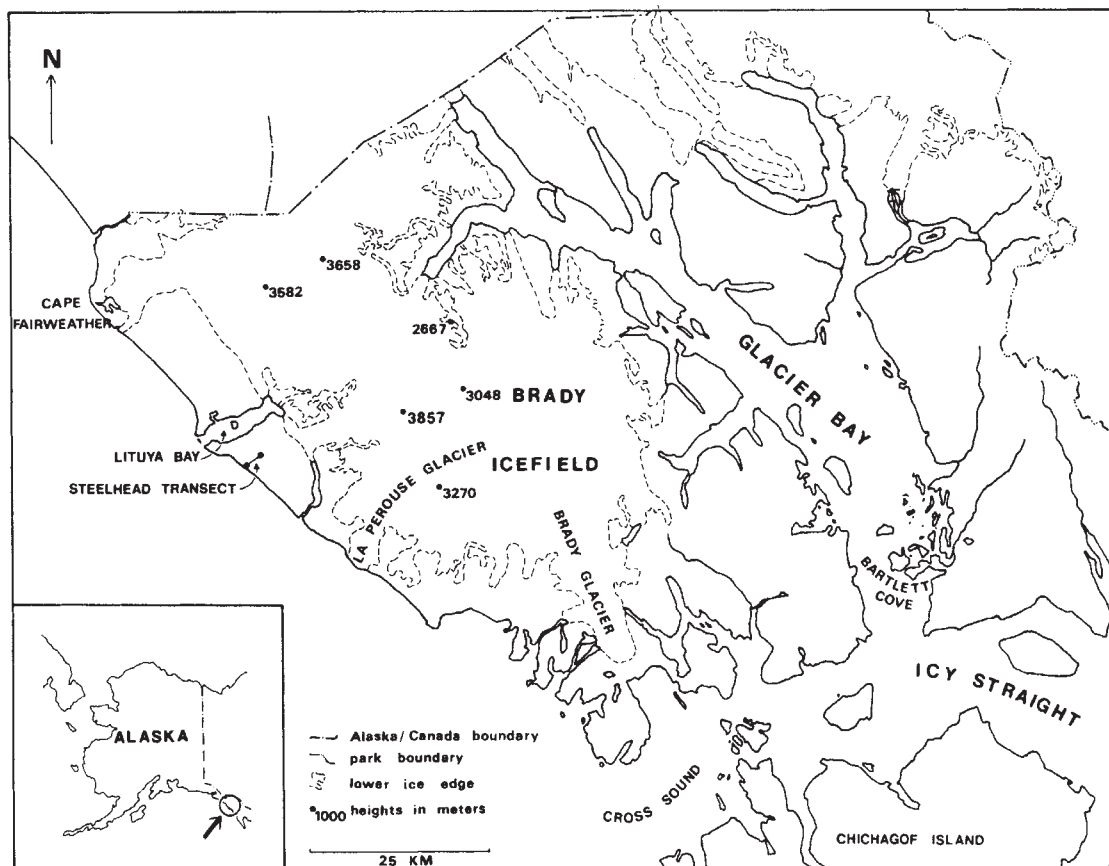
The seaward edge of terrace A is covered by rye grass (*Elymus arenarius*) and supports immature soils with A/C sequences (regosols or typic udipsamments). A Sitka spruce (*Picea sitchensis*) forest occupies the inland side of this terrace, and the soil has weakly developed B horizons with A(B)C profiles.

The seaward side of terrace B is covered by Sitka spruce with western hemlock (*Tsuga heterophylla*) in the understory. Soils on the seaward side are mature typic haplorthods or placorthods (podzols) with an A2, a friable B2hr and a firm or cemented B2ir horizon. The inland side of terrace B is dominated by western hemlock and Alaska yellow cedar (*Chamaecyparis nootkatensis*). The soils exhibit thick organic horizons, impervious B horizons (placic horizons) and impeded drainage.

Alaska yellow cedar and mountain hemlock (*Tsuga mertensiana*) form a stunted forest on the seaward edge of terrace C. Soils in this forest are histosols (peat soils) with some spodic (podzolic) characteristics, but the organic horizons are in excess of 1 m and extremely poorly drained. The rest of terrace C is occupied by peatland vegetation dominated by sphagnum, sedges and ericaceous shrubs growing on 1.5–2.0 m of well decomposed peat. This peat lies on a thin (15 cm), compacted, iron-stained layer, rich in woody organics, resting on sand which shows no morphological or chemical evidence of B2hr or B2ir horizons. Biotic remains in this profile indicate a successional change from forest to peatland. The basal woody layer contains remains of a carabid beetle, *Patrobus fossifrons*, which normally does not inhabit sphagnum bogs⁶; abruptly above this level seeds of the bog plant *Menyanthes trifoliata* appear.

The successional sequence is further described by the following chemical, physical and biological parameters of the soil. With distance from the sea, the organic horizon thickness increases from 2 to 20 cm across terrace A and from 22 to 35 cm across terrace B. On terrace C, a 100-fold increase occurs across the stunted forest/peatland boundary. The abundance of collembola and mites in organic horizons provides an index of the decomposition due to aerobic organisms. These soil arthro-

Fig. 1 Map of Glacier Bay National Monument, Alaska, showing the Lituya Bay and the location of study site.



Pods disappear rapidly inland along the transect, reaching lowest numbers in the stunted forest of terrace C. The pH decreases with increasing age and development of the soil. It decreases 2.5 units between the beach meadow and the bog, the greatest drop occurring at the start of the conifer forest. The amount of iron in the B horizon, as crystalline, inorganic amorphous, and organically complexed, parallels the soil development; it increases from 0.16% in the beach meadow to a maximum of 1.84% in the B2hr of the seaward side of terrace B. It decreases rapidly on terrace C as the soils become more poorly drained. The organically complexed iron increases from 0.60% in the B2 horizon at the rear of terrace A to a maximum of 1.00% in the B2ir of the seaward side of terrace B. Such iron is progressively depleted at the rear of terrace B and on terrace C. Virtually the only form of iron left in the profile at the bog site is organically complexed and associated with the basal layers of woody peat. The log of saturated flow of intact soil cores decreases from $1 \text{ ml min}^{-1} \text{ cm}^{-2}$ in the B2 of terrace A to $-3.5 \text{ ml min}^{-1} \text{ cm}^{-2}$ in the B2hr of terrace C. Field observations revealed an abundant flow of water along the surface of the B2hr in several profiles on terraces B and C.

The above observations clearly show that the terraces near Lituya Bay comprise a soil and vegetation chronosequence spanning about 8,000 yr and telescoped into an horizontal distance of less than 1 km. The rapid rates of both soil formation and plant succession are favoured by the maritime climate of South East Alaska which allows an unusually long growing season for this latitude⁷. Heavy precipitation (280 cm) and low evapo-transpiration encourage lush plant growth and rapid mineral weathering.

The terrace chronosequence near Lituya Bay offers a new perspective on the interaction between soil development, paludification and plant succession in South East Alaska. We propose the following sequence of events for peatland development on the terraces south-east of Lituya Bay (Fig. 3). (1) Soil-forming processes initiate the development of genetic horizons on surfaces newly emerged from the sea. (2) The

accumulation of sesquioxides in the B horizon, induced by podzolisation, results in the formation of plagic horizons. In South East Alaska plagic horizons are known to appear after about 500 yr⁸. (3) Plagic horizons cause deterioration of the soil's internal drainage, creating anaerobic conditions in the overlying horizons with attendant lowering of the decomposition rate of organic matter in the forest floor. Field observations show that plagic horizons form an effective barrier to plant roots. (4) Because of slowed decomposition, litter accumulation on the forest floor proceeds rapidly. (5) At some point, equivalent to the stunted forest zone of terrace C, the litter layer surpasses a critical mass and becomes capable of retaining water the whole year round to maintain anaerobic conditions without the help of impervious B horizons. (6) In the face of continuous anaerobic conditions, the iron-cementing agents of the plagic B horizons become reduced and removed in the groundwater. (7) Organic acids have lowered the pH and conditions are right for

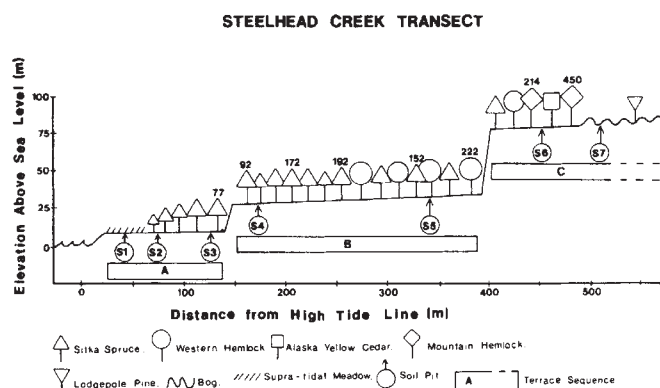


Fig. 2 Cross-section of the Steelhead Transect showing soil pit locations and tree ages across the raised terraces.

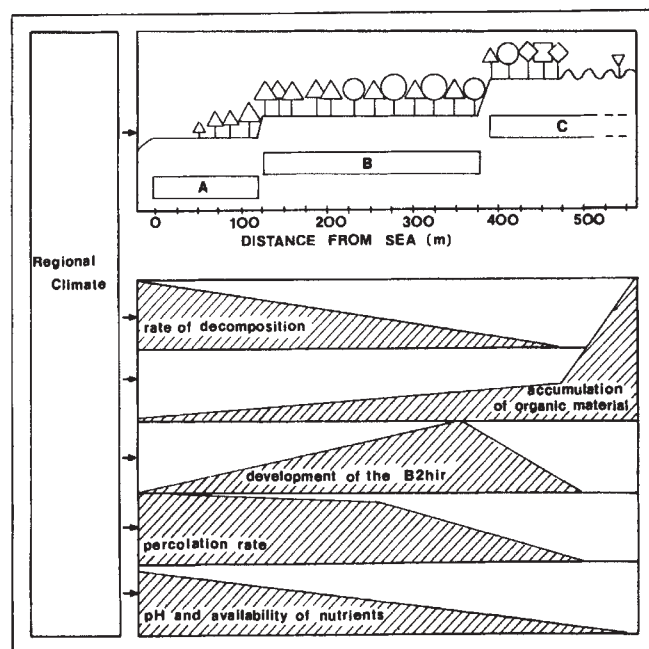


Fig. 3 A model of bog formation near Lituya Bay, South East Alaska.

the succession of bog vegetation onto a surface which once supported a well drained spruce forest.

Along this chronosequence of marine terraces, forest paludification is seen to accompany the development of spodosols. Iron cementation of B horizons, thickness of organic horizons, and hydraulic conductivity in these soils suggest a causative role for placic horizons in the paludification process. Other possible mechanisms of bog formation such as lake-infilling or the presence of impermeable parent material can be eliminated as alternative causes of paludification at this site. The spodosols responsible for paludification disappear in the anaerobic conditions occurring once the bog is established. Hence, little evidence for a pedogenic origin is available under existing bogs; a surface chronosequence seems necessary to document this process.

The role of soil development in peatland formation has been mentioned but seldom documented in the literature, and never previously assigned a major role in widespread paludification⁹⁻¹¹. The data and model presented above suggest that pedogenically induced bog formation may be more common in South East Alaska and perhaps in other regions of the world than previously recognised.

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Behavioural access to short-term memory in bees

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Memory formation proceeds in temporal phases which differ in their effectiveness in controlling subsequent behaviour and in their susceptibility to amnesic treatments¹. The initial phase of memory formation, frequently termed short-term memory, is generally considered a necessary precursor to long-term memory. However, the course of short-term memory differs widely between animal species and is dependent on experimental procedure. Information may even bypass the short-term phase en route to the long-term one^{2,3}. Experiments reported here using honey bees in a behavioural learning situation suggest that the greatest significance of short-term memory is its function as a mode of memory storage which may be altered effectively by new and contradictory information. Freely flying honey bees were presented two colour alternatives and rewarded on first one and then the other in a reversal learning paradigm. Subsequent colour preference was dependent on the interval between the two trials. Several new features of short-term memory are described. It is concluded that a single mechanism of short- to long-term memory transfer cannot account for the observed bimodal interval dependent behaviour. Two mechanisms are proposed.

The experimental procedure resembled that used in previous studies in which freely flying bees were taught to associate food with a colour stimulus²⁻⁵. Several bees are trained to use a feeding station. A newly recruited bee is marked as the experimental animal and given three pretraining trials to a neutral grey background. Two coloured cards, blue and yellow, are then presented and spontaneous choice test used to establish that the bee has no colour preference. Actual training starts by rewarding the bee once on one of the two colours. Choice behaviour is tested after each reward. The experimental protocol is illustrated in Fig. 1. The first reward is on blue, the second on yellow. A reversed order of colour training gives the same results. Time interval between these trials is varied between 10 s and 10 min. This scheme tests the effects of information initiated by the second trial on that stored during the first. In addition, the effects of temporal separation of the two training trials are also elucidated. Previous results^{2,3,5} suggested that the information capacity of short-term memory is both limited and time dependent. Thus variation of the inter-trial interval is a tool to describe short-term memory by a simple behavioural method. Another test after a second reversal learning trial gives information on the stability of these effects. Results are shown in Fig. 2.

Spontaneous choice activity was 50%. The results of learning and reversal learning in the long term are shown by arrows on the right ordinate of Fig. 2; reward duration was 60 s, and the interval between learning and reversal trials 10 min. The time course of interference between initial and reversal learning in the short term is examined by shortening the inter-trial interval. This is accomplished by decreasing reward duration to only a few seconds, making intervals as short as 10 s possible. The percent of choice behaviour has been shown to be independent of both duration and quantity of reward^{2,6}. Accordingly, reward durations of 5 and 15 s were used. In both situations there was a dominant reduction in response to the initially rewarded blue colour mark for inter-trial intervals of 3 to 5 min. During this period information from the second trial, the first reversal trial

on yellow, dominates the choice behaviour. At both greater and lesser inter-trial intervals choice behaviour was dominated by information from the initial trial on blue.

The results may be interpreted as follows. A learning trial initiates a short-term memory phase, which terminates 3–5 min later, and is followed by a consolidation phase. During the initial period of short-term memory and later with increasing consolidation new and contradictory information does not reach the memory store since the short-term memory and the consolidation process are already occupied by the information coming from the initial trial. In the transitional phase between short-term memory and consolidation, however, the information is more labile; it controls the choice behaviour less accurately^{2,3,4} and more readily accepts new and contradictory information. This interpretation is in agreement with previous results⁷ in which subjection of bees to electroconvulsive shock, cooling or N₂ and CO₂ narcosis disrupted memory only during the first few minutes after the learning trial. Since response levels in sessions A and B are similar and response functions from the first and second tests are parallel, the inter-trial interval must be the crucial parameter. No additional previously cryptic learning is expressed in the second test.

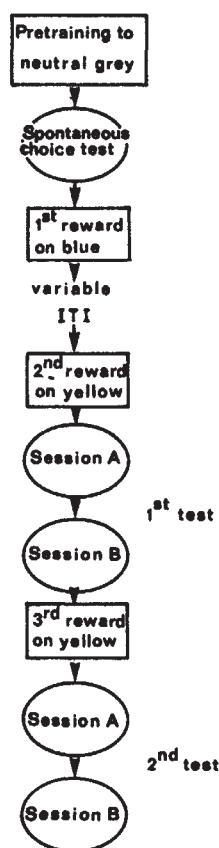


Fig. 1 Experimental scheme. Training sessions are shown in rectangles, and tests in ellipses. Single, individually marked foraging bees (*Apis mellifera carnica*) are pretrained to grey cards and then tested for spontaneous choice between blue and yellow cards presented simultaneously. All cards are 7 cm in diameter. The first training reward is on blue, the second on yellow and the third on yellow. ITI (inter-trial interval) is defined as the period between the first and second training trials. Tests consist of two sessions, A and B. Each session lasts 4 min. Time interval between A and B is 3–6 min. Three classes of choice behaviour are monitored: (1) approaching the colourmarks, (2) touching colourmarks with antennae and/or legs, and (3) landing on colourmarks. Approach data are presented here. Touching and landing data show similar results.

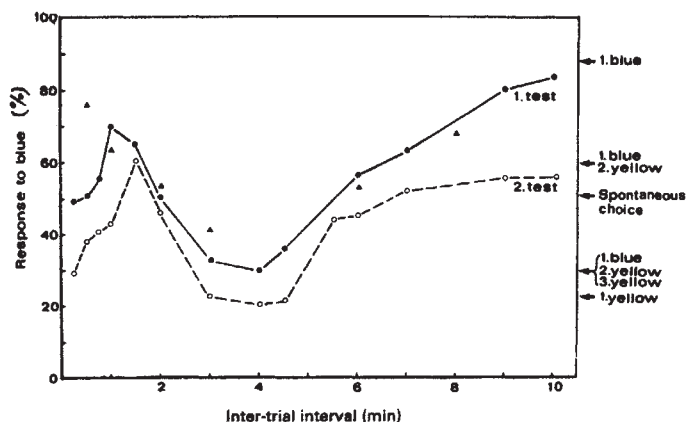


Fig. 2 Inter-trial interval and reversal learning. Results of sessions A and B are similar and, therefore, pooled. Circles are used to illustrate experiments with 5-s reward durations (no. of test animals 57; no. of choices 1,493); triangles represent experiments with 15-s reward duration (14 bees, 278 choices). Arrows on right ordinate: 1. blue, response level after one reward on blue (60-s reward duration); 1. blue 2. yellow, response level after a first reward on blue and a second on yellow (60-s reward duration); 1. blue 2. yellow 3. yellow, response level after a first reward on blue, a second on yellow and a third on yellow (60-s reward duration); 1. yellow: response level after one reward on yellow. In all cases averaged responses of five to eight bees are given. Differences of averaged responses of 15% or more are significantly different at the ≤ 0.01 level (χ^2 test, 2×2 table).

Our hypothesis does not account for the results obtained when the two training trials are presented within 1 min of each other. In this case, short-term memory should be strongest immediately after the trial^{2,5,7}. However, for such short inter-trial intervals the bees switch to the second colour (see Fig. 2, first min of inter-trial interval). Furthermore, if bees are rewarded for 15 s (triangles in Fig. 2) there is not such an early reversal effect. We interpret these findings to indicate an additional mechanism which acts only during the period immediately following the initial trial and 'erases' the information just stored in the short-term memory by the action of new, contradictory information. This mechanism works only if contradictory information arrives in the period immediately following trial. Identical information in massed learning trials adds to each other and can even reach long-term memory directly³.

Thus there are two temporal periods where reversal of learned behaviour is facilitated: The first ($t \leq 1$ min) is during the assimilation of the short-term phase, the second ($3 < t < 5$ min) is during the transition from short-term to consolidation phases. Information is eliminated most effectively if it has not yet been stored firmly in either the short-term or the long-term phase. The biological function of short-term memory is therefore not limited to that of a precursor for long-term memory; more importantly it is a mode of memory storage which allows for refinement by new incoming information.

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An unusual benzazocine elicits acetylcholine release in the isolated guinea pig ileum

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UM-1037 (3-allyl-1, 2, 3, 4, 5, 6-hexahydro-8-hydroxy-6-methyl-3-benzazocine), a benzazocine compound related structurally to the narcotic antagonists cyclazocine and the *N*-allyl analogue of cyclazocine, SKF-10,047, but lacking the 2,6-methano bridge, has been found to produce similar signs in normal rhesus monkeys to those characteristic of the narcotic abstinence syndrome¹. Narcotic antagonists have been reported to produce contraction in the isolated guinea pig ileum^{2,3}. However, only when the ileum has been continuously exposed to morphine or related drugs *in vitro* or has been isolated from animals made dependent on a narcotic, does it contract when an antagonist such as naloxone is added to the perfusion medium⁴. Thus, this preparation has been used as an *in vitro* model of the narcotic abstinence syndrome, and the contraction might be considered an 'abstinence sign'. In the present study, UM-1037 was found to produce a contraction of the guinea pig ileum isolated from normal animals similar to the contraction produced by naloxone in preparations exposed to morphine or related drugs. UM-1037, like naloxone, seems to act by releasing acetylcholine (ACh), because its actions were antagonised by atropine and tetrodotoxin. However, morphine, which is necessary for the naloxone-induced contraction, reversed the effect of UM-1037 on the ileum. Responses to UM-1037 were not altered in the presence of naloxone. Thus, the release of ACh which is produced by this unusual benzazocine in the ileum does not seem to be mediated by the opiate receptor.

The isolated guinea pig ileum was prepared as described by Paton⁵. Segments of ileum were suspended in a Krebs physiological buffer at 37 °C, saturated with 95% O₂-5% CO₂. The buffer contained NaCl, 118 mM; KCl, 4.75 mM; CaCl₂·2H₂O, 2.54 mM; MgSO₄·7H₂O, 1.19 mM; KH₂PO₄, 1.19 mM; glucose, 11 mM; NaHCO₃, 25 mM; hexamethonium Br, 0.07 mM; pyrilamine maleate, 0.125 μM. Resting tension on the tissue was 0.5 g. Tissues were equilibrated for 30–40 min with washes every 10 min. After the equilibration period, ACh, 5 × 10⁻⁶ M, was added to the bath to determine the maximum contraction of the tissue. Ten minutes after washing the ACh from the organ baths, cumulative concentration-response relationships were determined for UM-1037 by increasing the concentration of the drug by three-fold increments until a maximum response was obtained. After washout of UM-1037, ACh, 5 × 10⁻⁶ M, was added once again to determine whether the contractility of the preparation had changed.

Addition of UM-1037 to the organ bath produced a contraction of the isolated ileum (Figs 1, 2). The maximum contraction produced by UM-1037 was 53.5 ± 8.1% of the contraction produced by ACh, 5 × 10⁻⁶ M, and ED₅₀ for UM-1037 was 5.33 ± 0.97 × 10⁻⁶ M (*n* = 8). The effect produced by UM-1037 in the isolated ileum not exposed to morphine-like drugs resembles that produced by naloxone in preparations exposed to such drugs².

As the contraction produced by naloxone is inhibited by atropine, 10⁻⁷ M, and tetrodotoxin, 2 × 10⁻⁷ M, it is thought to be caused by a release of ACh from postganglionic cholinergic nerve terminals in the myenteric plexus⁶. To determine whether the contraction produced by UM-1037 was due to a similar mechanism, we investigated the effects of atropine and tetrodotoxin on this response. Atropine, 10⁻⁸ M or 10⁻⁷ M, added to the organ bath 20 min before UM-1037, antagonised the effects

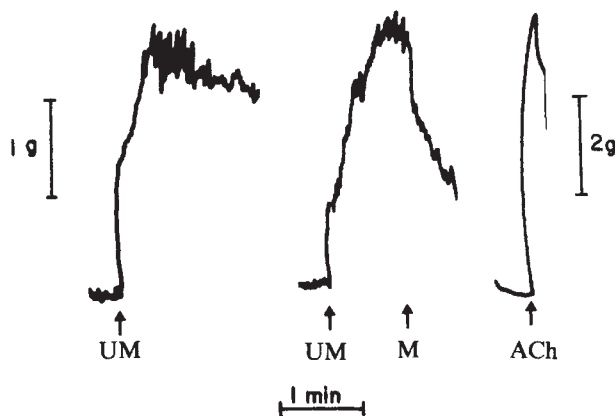


Fig. 1 Effects of UM-1037 on the isolated guinea pig ileum and antagonism by morphine. UM-1037, 3 × 10⁻⁵ M, was added to the organ bath at the time indicated by arrow UM. Morphine, 10⁻⁷ M, was added at arrow M. After washing the preparation, ACh, 5 × 10⁻⁶ M, a maximally effective concentration, was added to the organ bath at arrow ACh.

of UM-1037 (Fig. 2). In the presence of atropine, the concentration-response curve for UM-1037 was shifted to the right, and the maximum response was decreased. Tetrodotoxin, 2 × 10⁻⁷ M, also blocked the response of the ileum to UM-1037 when added 10 min before UM-1037 (Fig. 2). Although this concentration of tetrodotoxin inhibited the electrically induced contraction of the ileum, it did not affect the response to ACh. The interactions with atropine and tetrodotoxin suggest that UM-1037, like naloxone, produces a contraction of the tissue by releasing a limited store of ACh from cholinergic neurones; this in turn acts on the muscarinic receptors of the intestinal muscle.

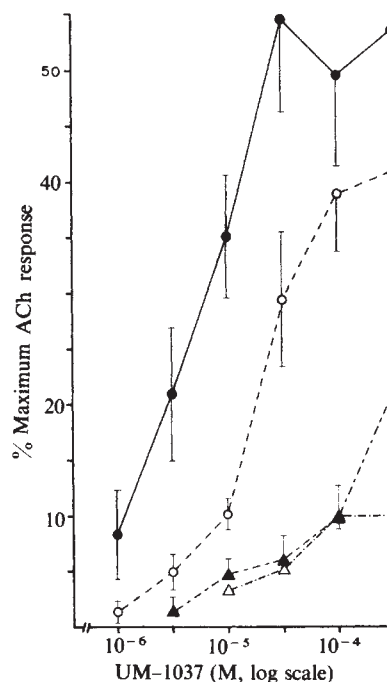


Fig. 2 Antagonism of UM-1037 by atropine and tetrodotoxin. Ordinate, per cent maximum contraction produced by ACh, 5 × 10⁻⁶ M. Abscissa, molar concentration. Concentration-response curves for UM-1037 in the guinea pig ileum are shown: eight control ilea (●—●); five ilea in the presence of atropine, 10⁻⁸ M, added to the bath 15 min before UM-1037 (○---○); five ilea in the presence of atropine, 10⁻⁷ M, added to the bath 15 min before UM-1037 (▲---▲); three ilea in the presence of tetrodotoxin, 2 × 10⁻⁷ M, added to the bath 10 min before UM-1037 (△---△). Mean values are given; vertical lines represent s.e.m.

Morphine-like agents inhibit the release of ACh from post-ganglionic cholinergic nerve terminals in the myenteric plexus of the guinea pig ileum^{7,8}. This ability to inhibit ACh release is correlated with the ability to bind with specific receptors in the ileum⁹. To determine whether UM-1037 acts at specific opioid receptors, the interaction between UM-1037 and morphine was studied. Morphine, 10^{-7} M, reversed the contraction produced by a maximally effective concentration of UM-1037 (Fig. 1), and when added to the organ bath before UM-1037, it caused a parallel shift to the right in the UM-1037 concentration-response curve (Fig. 3). To ascertain whether muscarinic receptors in the ileum were affected by this concentration of morphine, the effect of carbachol was studied in the presence and absence of morphine. The ED_{50} values for carbachol alone, and when morphine, 10^{-7} M, had been added 5 min previously, were $1.15 \pm 0.09 \times 10^{-7}$ M ($n = 4$) and $1.37 \pm 0.38 \times 10^{-7}$ M ($n = 6$), respectively. Therefore, morphine reverses the effect of UM-1037 at a concentration which does not block muscarinic receptors. The antagonism of the effect of UM-1037 by morphine as well as by tetrodotoxin and atropine, further indicates that the contraction is due to release of ACh.

The interaction between morphine and UM-1037 may be due to competition at the same receptor site or to opposing effects mediated by interactions of the drugs at different receptor sites. Naloxone, a competitive narcotic antagonist, was used to elucidate this interaction further. Concentration-response relationships for UM-1037, determined 5 min after the addition of naloxone, 10^{-7} M, to the bath, did not differ from those for UM-1037 alone (Fig. 3). This suggests that naloxone and UM-1037 occupy different receptor sites. Naloxone, when added 5 min before morphine, 10^{-7} M, prevented the reversal of the effect of UM-1037 by morphine (Fig. 3). Because naloxone competes with morphine for opiate receptor-binding sites, it is probable that the antagonism of UM-1037 by morphine is mediated through the interaction of morphine with the opiate receptor to decrease ACh release in the guinea pig ileum. These results suggest that the interaction between UM-1037 and morphine does not occur by competition at a common receptor.

To determine whether UM-1037 is a narcotic antagonist, the effect of morphine was studied in the presence and absence of UM-1037. The preparation was coaxially stimulated at 0.1 Hz and supramaximal voltage for 1 h. Then cumulative concentration-response relationships were determined for morphine alone and repeated on other segments of tissue when UM-1037, 10^{-6} M, had been added 5 min previously. UM-1037 decreased the maximum inhibition produced by morphine (24.4% decrease), but did not produce a parallel shift to the right of the morphine concentration-response curve. The ED_{50} values for morphine in the presence and absence of UM-1037, 10^{-6} M, were $2.97 \pm 0.55 \times 10^{-8}$ M ($n = 5$) and $2.33 \pm 0.97 \times 10^{-8}$ M ($n = 5$), respectively. These results are consistent with the concept that morphine and UM-1037 do not compete for common receptors, and that UM-1037 decreases the maximum effect of morphine in the guinea pig ileum through an interaction with another site.

Prior exposure to a narcotic is a requirement for antagonist-precipitated abstinence in animals as well as for the antagonist-precipitated contraction of the isolated guinea pig ileum. The mechanism of narcotic antagonist-precipitated abstinence is generally thought to involve displacement of the narcotic from receptor sites.

In this study, the effects of UM-1037 on the isolated guinea pig ileum were studied. In this preparation, as in the monkey, a sign considered to be characteristic of narcotic abstinence was produced in the absence of narcotic exposure. The effects produced by this drug in the isolated guinea pig ileum resemble those produced by naloxone in preparations exposed to morphine in that release of acetylcholine mediates the contraction of the tissue in both cases. Unlike the naloxone-precipitated abstinence sign in the ileum, the effect produced by UM-1037 is antagonised by morphine.

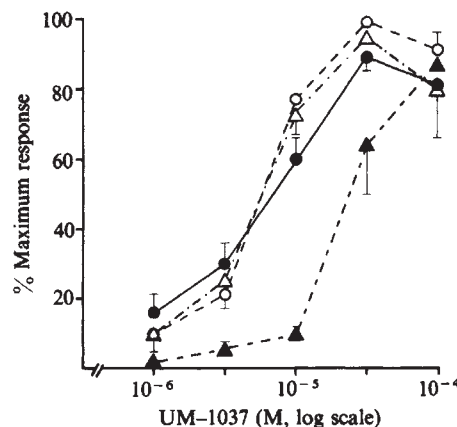


Fig. 3 Interaction between UM-1037, morphine and naloxone on the isolated guinea pig ileum. Ordinate, per cent maximum contraction. Abscissa, molar concentration. Concentration-response curves for UM-1037 in the guinea pig ileum are shown: eight control ilea (●—●); four ilea in the presence of morphine, 10^{-7} M, added to the bath 5 min before UM-1037 (▲—▲); three ilea in the presence of naloxone, 10^{-7} M, added to the bath 5 min before UM-1037 (○—○); four ilea in the presence of both naloxone, 10^{-7} M, added to the bath 10 min before UM-1037 and morphine, 10^{-7} M, added to the bath 5 min after naloxone and 5 min before UM-1037 (△—△). Mean values are given; vertical lines represent s.e.m.

A syndrome resembling the narcotic abstinence syndrome has been described in normal rats after the administration of phosphodiesterase inhibitors¹¹. This 'quasi-abstinence syndrome' is antagonised by narcotics and potentiated by naloxone. However, UM-1037 does not act like a phosphodiesterase inhibitor. Although morphine antagonises the effect of UM-1037 in the isolated guinea-pig ileum, naloxone does not alter the response to UM-1037. Furthermore, theophylline, a methylxanthine which is a phosphodiesterase inhibitor, does not produce a contraction of the isolated guinea pig ileum, although the methylxanthines do enhance the electrically induced contraction of the ileum¹². At high concentrations theophylline inhibits the twitch of the stimulated preparation¹². UM-1037 produces a contraction of the isolated ileum and does not inhibit the twitch at any concentration. Therefore, UM-1037 and phosphodiesterase inhibitors do not seem to share a common mechanism of action in this preparation.

Like UM-1037, nicotine and 5-hydroxytryptamine cause a release of ACh from cholinergic neurones in the isolated guinea pig ileum¹². Preliminary studies indicate that the actions of UM-1037 on this preparation are not mediated by either nicotinic or tryptaminergic receptors^{13,14}.

Although the present study suggests that the precipitation of the abstinence syndrome by narcotic antagonists in morphine-dependent ilea and the abstinence-like syndrome produced by UM-1037 in non-dependent ilea involve different receptor sites, it is possible that common mediators or neural pathways are involved in both drug effects. UM-1037 and related compounds may be important in elucidating the mechanism underlying the narcotic abstinence syndrome.

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Curare has a voltage-dependent blocking action on the glutamate synapse

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The skeletal muscle of members of Orthoptera and Diptera receives an innervation which is probably glutaminergic¹⁻³. Recent study of the ventral muscle fibres in the larvae of the beetle, *Tenebrio molitor*, has revealed that the transmitter action can be mimicked by the iontophoretic application of L-glutamate to the junctional sites at which the extracellular excitatory postsynaptic potentials (e.p.s.ps) could be recorded (D.Y. and H.W., unpublished observation). Contrary to the evidence favouring glutamate as a transmitter of junctional excitation in insects, some investigators have found that curare (+)tubocurarine, TC, a classic acetylcholine (ACh) antagonist, suppresses the neurally evoked muscle potentials in the fly *Sarcophaga*^{4,5}, and *Tenebrio*⁶. Here, we have analysed the action of curare on the neuromuscular junction of *Tenebrio* larvae and found that curare blocked the glutaminergic transmission by antagonising the transmitter at the postsynaptic site.

The dissection used has been described previously⁷. The ventral muscle fibres were penetrated by two microelectrodes, one for recording and another for passing current, filled with 3 M KCl and 2 M K citrate, respectively. When necessary, the muscle fibres were voltage clamped using a two-microelectrode method⁸. For focal extracellular recording, electrodes were filled with 3 M NaCl. Although the signals recorded extracellularly could be resolved into single traces on the oscilloscope, a minicomputer (Sanei signal processor 7T07A) was used to obtain a signal averaging so as to improve the signal-to-noise ratio. The iontophoretic electrodes were filled with 1 M solution of monosodium L-glutamate (pH 8.0). The e.p.s.ps were evoked by stimulating the innervating nerve close to the segmental ganglion. All experiments were carried out in a low Ca²⁺ saline solution to any minimise movement artefacts accompanying an action potential. The standard bathing saline solution had the following composition (mM): NaCl, 70; KCl, 30; CaCl₂, 0.8; MgCl₂, 14.2; glucose, 445; HEPES, 5. The pH of the solution was adjusted to 7.2 with NaOH.

Curare, when applied to the bathing solution at concentrations higher than 5×10^{-4} M, reversibly depressed the e.p.s.p. amplitude without affecting either the input membrane resistance or the resting potential of the muscle fibre. Simultaneous focal recordings have revealed that the postsynaptic current was substantially depressed but that the presynaptic impulse and the synaptic delay remained essentially unchanged (Fig. 1). The quantum content estimated by the failure method⁹ was unaffected by the application of curare (Table 1). These facts indicated that the site of action of curare was on the postsynaptic membrane. The observations do not necessarily mean that the presynaptic terminal of *Tenebrio* neuromuscular junction lacks

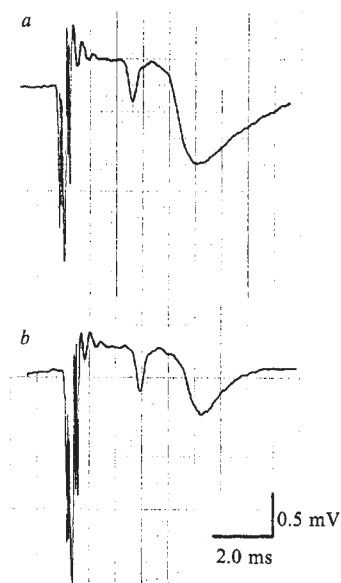


Fig. 1 The nerve terminal action potential and the e.p.s.p. recorded extracellularly from the neuromuscular junction. Records were taken before (a) and 5 min after (b) bath perfusion of 7.5×10^{-4} M curare. Signals were averaged over 99 trials.

the ACh receptors reported to be present on the locust junction¹⁰, because curare failed to affect transmitter release even in the locust system unless it was applied simultaneously with ACh^{10,11}.

To characterise the curare-induced suppression further, we examined the action of curare on the glutamate potential. We found that the amplitude of the glutamate potential decreased and the time to peak was markedly prolonged after treatment with 5×10^{-4} M curare. One possible explanation for the prolonged glutamate potentials in TC solution is that the L-glutamate molecules ejected must diffuse over a greater distance to reach the sensitive receptors than would be required in normal conditions because some accessible receptors which are distributed on the fibre surface may become unavailable through the action of TC. Both effects of curare on the glutamate potential were fully reversible. Curare also produced a reduction in the apparent maximum of the dose-response curve for glutamate-induced depolarisation (Fig. 2). The non-competitive

Fig. 2 Effect of curare on the dose-response curve to glutamate recorded from a single mealworm muscle fibre: Results are of glutamate-evoked membrane depolarisation measured in control solution (●) and in 7.5×10^{-4} M curare (○).

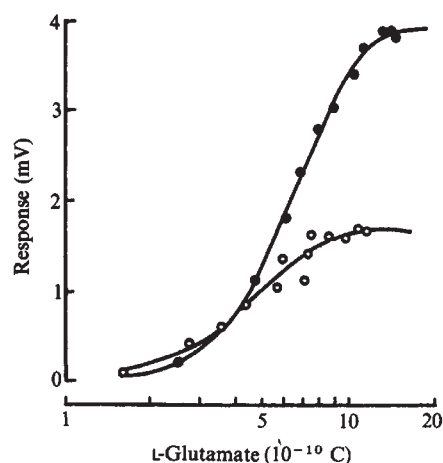


Table 1 Effect of curare on quantum content

Expt	Concentration (mM)	Before TC	During TC	Quantum content ratio (during/before)
1	0.75	2.15	2.03	0.94
2	0.75	1.74	1.56	0.90
3	0.5	1.03	0.90	0.87
4	0.5	1.41	1.82	1.29
Mean and s.e.		1.00 ± 0.098		

Quantum content was calculated from the number of failures of extracellular e.p.s.ps. The number of stimuli ranged from 198 to 396; the stimulation rate was 5 Hz.

antagonism has been taken as evidence that the drug blocks ionic channels mediated by the transmitter rather than acting on the receptor itself¹². Furthermore, there is additional evidence supporting the notion that curare is acting on the glutamate channel. Thus, in contrast to its normal behaviour, the amplitude of the glutamate current under voltage clamp does not increase with hyperpolarisation, but may actually be reduced (Fig. 3). At cholinergic synapses in *Aplysia* neurones¹³ and frog muscle¹⁴, curare has been shown to produce a negative slope in the membrane potential–ACh current relationship with hyperpolarisation, but the amplitude of the excitatory postsynaptic current (e.p.s.c.) increased linearly with hyperpolarisation even in the presence of curare ('Manalis effect' (ref. 15)). Contrary to results in these cholinergic synapses, curare influenced the membrane potential–e.p.s.c. relationship in a similar manner to that observed in the iontophoretically induced responses in the present system. Representative families of e.p.s.cs before and during application of curare are shown in Fig. 4. In this respect, the action of curare on the glutamate-mediated junction resembles that of local anaesthetics on the cholinergic synapse. For example, procaine produces a non-linearity of the membrane potential–e.p.s.c. amplitude relationship in the frog neuromuscular junction¹⁶, suggesting that the drug becomes more effectively attached to its binding site in the activated receptor–mediator complex during hyperpolarisation. As in the case of procaine blockade of the cholinergic synapse, curare might combine with the receptor–transmitter complex, thereby

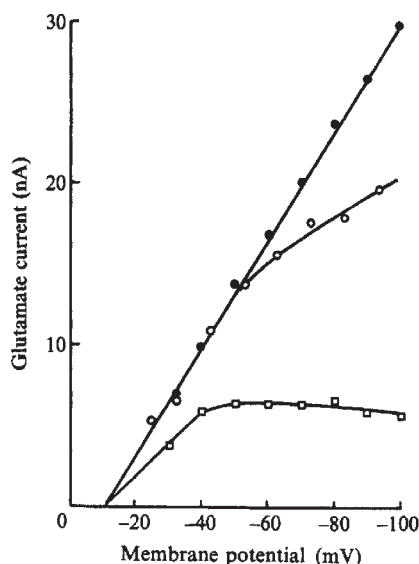


Fig. 3 Voltage dependence of the amplitude of the glutamate current measured before (●) and 5 min after (□) perfusion of 7.5×10^{-4} M curare and 15 min after washing with drug-free saline (○). The recovery after washing is incomplete in this fibre.

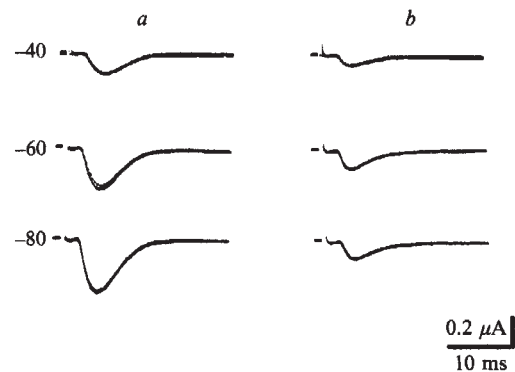


Fig. 4 Excitatory postsynaptic currents recorded at three different holding potentials (indicated at the left side of the each trace) before (a) and during (b) application of 7.5×10^{-4} M curare. Three successive e.p.s.cs evoked by repetitive stimulations at 0.5 Hz were superimposed on each record. Downward deflections indicate inward currents.

'plugging' the open channel itself^{15,17,18}. Alternatively, the present results showing an effect of curare on the e.p.s.c. as well as on the glutamate current may favour a model¹⁹ in which local anaesthetics can bind to the receptors before ACh does.

The blockade of non-cholinergic transmission by curare has already been reported in *Aplysia* neurones^{20,21}—depolarisations induced by dopamine and serotonin as well as Cl-dependent hyperpolarisation caused by serotonin were suppressed by curare. In addition, K⁺-dependent hyperpolarisations induced not only by dopamine and serotonin but also by ACh were completely insensitive to curare. Therefore, it seems possible that curare acted on the ionic channels rather than on the receptors in *Aplysia* neurones.

The present experiments demonstrate that the site of action of curare in *Tenebrio* ventral muscle is on the subsynaptic membrane. However, there is no evidence that curare has similar effects on synapses in other *Tenebrio* muscles. Reports that curare is inactive at synapses on some other insect muscles, such as *Periplaneta*²² and *Schistocerca*²³, probably reflect a species difference. The results are against the explanation that curare can indirectly depress the muscular electrical potentials by blocking conduction in the motor nerves^{24–26}. The study also suggests that results using curare as a pharmacological indicator of cholinergic involvement should be interpreted with caution.

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Recycling of noradrenergic storage vesicles of isolated rat vas deferens

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Del Castillo and Katz established the theory of quantal release of acetylcholine from the neuromuscular junction¹. The concept was expanded by showing that quanta of transmitter was packaged in, and released from, storage vesicles of the nerve endings. Noradrenaline (NA) is also believed to be stored in, and released from, storage vesicles of the sympathetic nerves. The requirement of Ca for NA release² and concomitant secretion of the vesicular enzyme, dopamine- β -hydroxylase³, on stimulation lends strong evidence to the theory of exocytosis. However, it remains unclear whether storage vesicles after fusing with neuronal membrane to release NA into cleft, are once again reused for further storage and release of NA, or are discarded by the nerve endings. I have investigated this question by making use of the fact that tetraethylammonium (TEA) causes profound facilitation of the overflow of ³H-NA when the sympathetic nerves of the isolated vas deferens are electrically excited⁴, and that this excess release is accompanied by a significant reduction in tissue NA content⁵. The principal advantage of the method is that the reduction in tissue NA content is effected exclusively by the exocytotic release of NA from the nerve terminals and easy washout of TEA allows one to study uptake of exogenous NA by the tissue. The results presented here support the hypothesis that NA storage vesicles are recycled.

Male rats weighing 300–400 g were killed by a blow on the head, and the vasa deferentia were removed. Vasa deferentia were placed in 1 ml Krebs solution gassed with 95% O₂–5% CO₂, and incubated at 37 °C for 30 min, and then incubated for 15 min with 0.35 μ M ³H-labelled ($\pm$)NA (specific activity, 13 Ci mmol⁻¹, Amersham Searle). Following termination of incubation, the tissues were washed three times with Krebs solution over a 30-min period, at the end of which tissues were removed to study either NA content and ³H-NA accumulation or the overflow of ³H-NA upon transmural stimulation as described previously⁶. In the case where repetitive stimulation was used for longer periods, a train of 150 impulses at 5 Hz was delivered every 30 s of 1 min for varying periods of time (80 V, 1-ms duration) from a Grass stimulator, Model S-88. In the case where overflow of ³H-NA was studied before and after TEA and repeated stimulation, the vas deferens was stimulated by a train of 900 impulses at 30 Hz. To obtain net overflow of ³H-NA released by stimulation, the amount of radioactivity spontaneously released within the immediately preceding equivalent time period was subtracted from the total amount released during the stimulation period⁶. Overflow of ³H-NA is expressed as a fraction of the total ³H-NA content of the tissue at the onset of stimulation. Endogenous NA content of the vas deferens was analysed fluorometrically⁷ as described earlier⁸. The same method was used to separate ³H-NA and ³H-catechol metabolites from the tissue bathing medium as previously described⁶.

As TEA enhances stimulation-induced overflow of radioactivity in the ³H-NA-labelled rat vas deferens by about 20-fold at 5 Hz, as compared to a four- to five-fold increase with phenoxybenzamine⁴, TEA was used to study its effect on tissue NA content at rest and during nerve activity. As shown in Table 1 the NA content of right vas deferens exposed to TEA was comparable to that of the contralateral control tissue. Intermittent transmural stimulation of the right vas deferens caused no significant change in the NA content. However, transmural

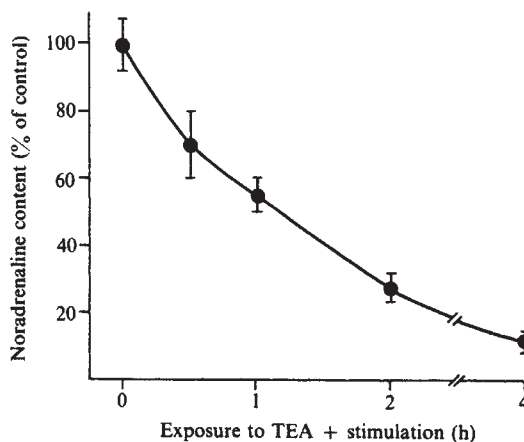


Fig. 1 NA content of the vas deferens various times after exposure to TEA and transmural stimulation. The right vas deferens was exposed to 10 mM TEA plus transmural stimulation (5 Hz, 30 s min⁻¹) for different time periods. The NA content of the treated tissue is expressed as a percentage of that found in contralateral control tissue ($11.6 \pm 0.1 \mu\text{g g}^{-1}$, $n = 20$) kept in 10 mM TEA–Krebs solution for a comparable time. Each point, except the 0-h point, represents a mean of 5 observations. Vertical lines show s.e.m.

stimulation in the presence of TEA significantly lowered tissue NA content. Figure 1 shows the combined effect of TEA and transmural stimulation on tissue NA content for different time periods. A significant reduction in NA content ($27 \pm 4\%$, $P < 0.01$) was found after 30 min and the degree of reduction increased with the exposure to TEA and transmural stimulation. A maximum reduction of 87% occurred after 4 h.

NA is equally distributed throughout the vas deferens⁸. Therefore, during the course of an experiment the tissue can be cut in several portions, each portion initially having comparable amounts of NA (or ³H-NA). Initial NA and ³H-NA contents present in a small portion of the vas deferens were $13.01 \pm 0.75 \mu\text{g g}^{-1}$ and $4,520 \pm 412 \text{ c.p.m. mg}^{-1}$, respectively (Fig. 2).

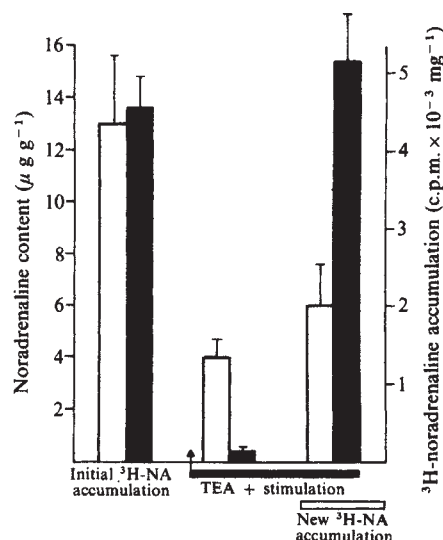


Fig. 2 Depletion and retention of ³H-NA before and after TEA and transmural stimulation for 2 h. NA content (open columns) and ³H-NA accumulation (solid columns) are shown in control tissue (first paired columns), in the same tissue after exposure to TEA plus stimulation for 2 h (Fig. 1) (middle paired columns), and in the same tissue after exposure to TEA plus stimulation for 2 h, and then incubation with ³H-NA (last paired columns). For details see text. Each column represents a mean of 5 experiments. Vertical lines show s.e.m.

Table 1 Effects of TEA and transmural stimulation, separately and together, on NA content of the vas deferens

Experimental conditions	No. of experiments	NA content		
		Control, left vas deferens	Experimental, right vas deferens	% Of control
		(µg g ⁻¹)		
TEA (10 mM, for 2 h)	5	10.06 ± 1.40	12.08 ± 1.70	120 ± 13 (NS)
Transmural stimulation (5 Hz, 30 s min ⁻¹ , for 2 h)	7	12.08 ± 1.70	12.80 ± 1.0	106 ± 9 (NS)
TEA + transmural stimulation	7	11.40 ± 0.8	2.90 ± 0.40	25 ± 6*

After 30 min equilibration of both vasa deferentia in Krebs solution, right vasa deferentia were exposed to either TEA, transmural stimulation, or TEA plus transmural stimulation for a total period of 2 h. Left vasa deferentia remained in Krebs solution (first 2 groups of experiments) or in 10 mM TEA (the third group) for 2 h to serve as controls. NS, not significantly different from the control.

* $P < 0.001$.

The remaining tissue was subjected to the combined treatment of TEA and transmural stimulation for 2 h. TEA was washed out for 15 min and the tissue was cut into two portions; one was again incubated with ³H-NA and subsequently washed, while the other remained in Krebs solution. TEA and stimulation lowered NA content by about 70%, and the ³H-NA level was 3% of the initial control. Such depleted tissue, when re-exposed to ³H-NA, took up and retained large quantities of ³H-NA. The new accumulation of ³H-NA was comparable to the initial accumulation by the control tissues not subjected to TEA and stimulation.

As maximum depletion of NA occurred at 4 h after treatment with TEA and stimulation (Fig. 1) the accumulation of ³H-NA in such severely depleted tissues was investigated. NA content was lowered from a control value of 9.54 ± 0.79 to 1.24 ± 0.3 µg g⁻¹, and ³H-NA was undetectable after TEA plus stimulation for 4 h. On re-exposure to ³H-NA, such depleted tissue retained ³H-NA in amounts identical to those found in control tissue before depletion ($4,190 \pm 396$ against $3,826 \pm 321$ c.p.m. mg⁻¹).

Cocaine was used to determine the sites of new accumulation of ³H-NA by the depleted vas deferens. In the control tissue, 3.6 µM cocaine reduced ³H-NA accumulation by 74% ($3,238 \pm 605$ against 862 ± 190 c.p.m. mg⁻¹, $n = 3$). Cocaine had a similar effect on ³H-NA accumulation in tissue initially depleted by TEA and stimulation ($3,260 \pm 388$ against 754 ± 173 c.p.m. mg⁻¹, $n = 3$).

Figure 3 shows the results of experiments in which overflow of newly acquired ³H-NA was studied in tissues previously subjected to TEA and stimulation. Following initial ³H-NA incubation and its washout, the overflow of ³H-NA induced by stimulation (30 Hz) averaged 17.9 ± 2.2 ($\times 10^{-4}$). TEA and transmural stimulation lowered ³H-NA levels to such an extent that no overflow from such tissues could be measured. However, on reloading of the tissue with ³H-NA, overflow induced at 30 Hz was almost identical to the initial overflow 18.7 ± 2.6 ($\times 10^{-4}$). The new overflow was depressed by over 90% in the absence of external Ca (Fig. 3b). Substitution of 2.5 mM Ca restored the new overflow to almost 75% of the initial control value.

As stimulation alone does not alter tissue NA content over a 2-h period, the amounts released through this method may be replenished by either the synthesis of new NA or uptake of released NA, or both of these mechanisms. With TEA and stimulation NA release must be so great that even accelerated synthesis together with reuptake cannot keep up with the release, resulting in a reduction in tissue NA content. Ca is needed for the TEA-induced facilitation of release during nerve stimulation. Therefore, the depletion of NA content of the isolated vas deferens must be a result of exhaustive exocytotic release of NA. The participation of newly synthesised storage vesicles can be totally excluded in this study, as this cannot be done in the case of the adrenal gland or sympathetic organ *in vivo*, because the hypogastling plexus innervating the vas deferens⁸ is not present in the preparation. Although certain

procedures reduce the NA content of the isolated tissues, these did not involve the exocytotic release of NA. It was shown that NA deprivation results in reduction in NA content of cat spleen slices⁹ and the rabbit heart¹⁰ without requiring external Ca. However, intermittent stimulation of splenic nerves of the dog in the presence of phentolamine caused significant loss of splenic NA content¹¹.

As it was shown that the amounts of ³H-NA retained by previously depleted vas deferens were almost identical to those retained by control tissues and as cocaine, a well-known inhibitor of neuronal uptake of NA¹², blocked initial and new accumulation of ³H-NA to the same extent, it is believed that the new accumulation occurs predominantly in the sympathetic nerves. If NA taken up by the neuronal membrane is not removed by the storage vesicles, then it is rapidly deaminated by intraneuronal monoamine oxidase¹³. In the present work it was found that even 30 min after washout comparable amounts of ³H-NA were retained by normal and depleted tissues. Therefore, it is assumed that ³H-NA taken up by depleted tissue is retained by the storage vesicles of the sympathetic nerve ending. Strong proof for the idea that the newly taken up ³H-NA is accrued to the storage vesicles is that stimulation releases newly acquired ³H-NA. It is known that if ³H-NA is not present in the storage vesicles, but is accumulated in extravesicular sites in the sympathetic nerves, it is not available for release by electrical stimulation¹⁴. The fact that newly acquired ³H-NA can be

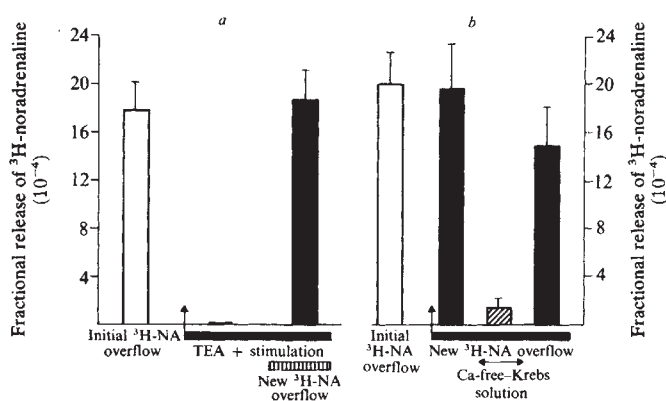


Fig. 3 Overflow of ³H-NA before and after TEA plus transmural stimulation for 2 h. Overflow of ³H-NA was induced by stimulating (30 Hz for 30 s) the initially labelled vas deferens (open column). Overflow of ³H-NE is expressed as fractional release which represents the ratio of the overflow of ³H-NA to that in the tissue at the beginning of the stimulation. The solid column represents the overflow elicited from relabelled tissues subjected to 10 mM TEA plus transmural stimulation in Krebs solution, as described above, then in Ca-free Krebs solution (hatched column), and finally in Krebs solution. *a*, Each column represents a mean of 8 experiments and in *b* represents a mean of 4 experiments. Vertical lines show s.e.m.

released in response to nerve stimulation, and that the new release is Ca dependent, would indicate that the release occurs by exocytosis.

The main question now is whether the same storage vesicles which participate in the storage and release of NA are responsible for the accumulation and subsequent release of ^3H -NA. As the maximum depletion of NA stores was 87% after TEA and stimulation, it may be that remaining storage vesicles which did not participate in exocytosis could take over the function of storage and release of the total original population of vesicles. Another possibility is that storage vesicles previously involved in exocytosis can be reused by sympathetic nerve terminals for the purpose of recapturing and releasing new NA.

It has been suggested that the membrane of the large noradrenergic vesicle in nerve terminals may be retrieved after

exocytosis in the form of small vesicles. The latter, then, might be the precursors of the functioning small vesicles¹⁵. In support of such an idea, it has been shown that newly formed small storage vesicles after nerve activity contain significant amounts of dopamine β -hydroxylase, and these vesicles are capable of storing ^3H -NA¹¹. An alternative suggestion is that after a vesicle fuses with the nerve membrane it becomes nonfunctional and is destroyed by the nerve¹⁶. Based on the experiments reported here, I feel that noradrenergic storage vesicles of the rat vas deferens can be reused more than once by nerve terminals. However, the lack of recovery of endogenous NA, following its depletion by exhaustive exocytosis, may be an indication that used vesicles lose their ability to synthesise NA and are only capable of storing preformed NA.

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A new neuronal marker identified by phosphorylcholine-binding myeloma proteins

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The difficulty of working with the intact brain *in vivo* has led to the increasing use of nerve cell cultures in neurobiology. However, dissociated cells cannot be unambiguously identified by morphological criteria before the third week in culture, for it is not until then that the basic morphology and size of neurones become stable so that these and other cell types can be easily distinguished¹. However, cultured neurones can be identified by various cytochemical techniques based on (1) the detection of neurotransmitters or receptors for transmitters^{2,4}, (2) the presence of the Thy 1 antigen and the receptor for tetanus toxin, which are present on the membrane of most neurones³, and (3) the presence in neurones of neurone-specific enolase (NSE), a cytoplasmic enzyme, which can only be identified on fixed specimens^{6,7}. Furthermore, other cell types in culture can also be specifically labelled. For instance, antisera to galactocerebroside bind selectively to oligodendrocytes⁸, and antibodies to a neural tumour bind selectively to Schwann cells^{5,9}. We report here the selective interaction of phosphorylcholine-binding myeloma proteins (PC-BMP)¹⁰ with mouse neurones in culture and in suspension. Phosphorylcholine (PC) is found as part of lecithin and sphingomyelin molecules in variable amounts in eukaryotic and prokaryotic membranes, including plasma membranes^{11–13}.

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Dissociated cells from the mouse (C57/B6) nervous system were cultured as previously described¹. Briefly, spinal cord, dorsal root ganglia or both, were dissected from 12–13-d embryos and cleaned of any adhering tissue. Excised tissue was cut into small pieces, trypsinised (0.25% in Eagle's minimal essential medium (MEM)) and further mechanically dissociated. Cells were seeded (5×10^5 cells per coverslip) on to 12-mm glass coverslips in 24-well plates (Cluster 24, Costar) coated with 0.05% collagen (Calbiochem) in 0.1% acetic acid. Cells were grown under 90% air and 10% CO₂ in bicarbonate-buffered MEM supplemented with 600 mg glucose per 100 ml, 10% heat-inactivated horse serum and, until the cultures reached confluency, 10% fetal calf serum. When confluent (at about 5–7 d), they were exposed for 2 d to 5-fluorodeoxyuridine (Sigma, 1.2 mg per 100 ml) and uridine (3 mg per 100 ml). This treatment, which prevents overgrowth of dividing non-neuronal cells, was repeated 2 weeks later if thought necessary. The medium was changed three times per week. Most experiments were carried out 4 weeks after plating, when the majority of cells could be identified by phase contrast microscopy on morphological criteria. These cultures were fixed with paraformaldehyde (1% for 5 min). Some experiments were carried out on freshly dissociated living cells in suspension.

The myeloma proteins used in these experiments included the following: TEPC 15; MOPC 167 and McPC 603, PC-BMP; XRPC 25, a dinitrophenol-BMP; EPC 109 and UPC 61, inulin-BMPs; XRPC 24, a β (1–6) galactan-BMP; and McPC 870, a lipopolysaccharide-BMP which also weakly binds PC¹⁰. All proteins were IgA κ and were purified by affinity chromatography, with the exception of McPC 870, which was isolated by ion-exchange chromatography followed by gel filtration. Goat anti-mouse IgA antibodies were raised, purified by affinity chromatography and tested as described elsewhere^{14,15}. The antiserum was passed over a mixed IgG (MOPC 195, MOPC 70 A, MOPC 173 myelomas)–Sepharose column and then eluted from an IgA (TEPC 15)–Sepharose column. The purified antibodies (GAM α) were coupled to fluorescein by reaction with fluorescein isothiocyanate (FITC)¹⁴ and a fluorescein: protein molar ratio of 4.5 was obtained. Rhodamine-coupled tetanus toxin (RTT) was prepared as described in ref. 16.

Fixed cells were exposed to MPs at concentrations up to 1 mg ml^{-1} for 1 h at room temperature, washed in phosphate-buffered saline (PBS) and incubated in GAM α (0.5 mg ml^{-1}) for 1 h at room temperature. For double-labelling experiments RTT, PC-BMP and GAM α were applied successively, each for 1 h at room temperature. Cells were mounted on glass slides for

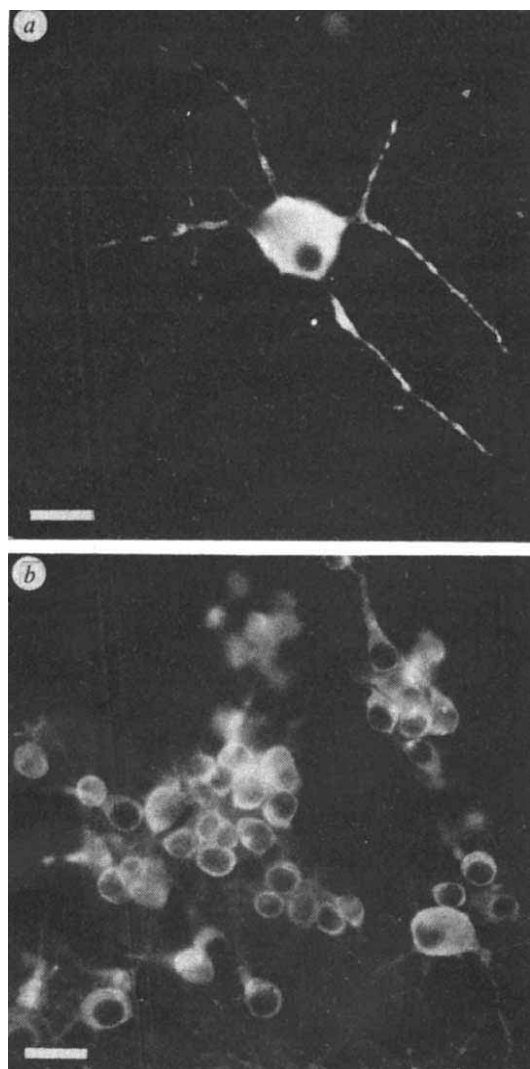


Fig. 1 Immunofluorescence of fixed mouse spinal cord and dorsal root ganglia cells. Four weeks after seeding, cells attached to coverslips were washed with serum-free MEM, fixed in 1% paraformaldehyde in PBS for 5 min at room temperature and washed extensively with PBS. The fixed cells were incubated with affinity-purified PC-BMP, in this case MOPC 167 (1 mg ml^{-1}), for 60 min at room temperature. After washing with PBS, they were incubated with fluorescein isothiocyanate-labelled goat anti-mouse α -chain antibodies (0.5 mg ml^{-1}) for 30 min at room temperature and washed with PBS. The coverslips were mounted on slides with 50% glycerol in PBS and were examined with a Zeiss microscope equipped with epi-illumination. *a*, Only one cell is labelled. It has the typical morphology of a neurone from dorsal root ganglia. The underlying and surrounding cells are negative. Scale bar, $33 \mu\text{m}$. *b*, A cluster of smaller spinal cord neurones and one probable dorsal root ganglionic cell are shown. Only neurones are labelled. Cell processes are also labelled, although they are not visible in this picture, where the plane of focus falls on the cell bodies. Scale bar, $40 \mu\text{m}$.

observation on a Zeiss microscope equipped with fluorescein and rhodamine optics. NSE was detected on parallel mature cultures as previously described⁷. Double-labelling with PC-BMPs and antibodies to NSE was not possible because of the severe conditions required for the detection of NSE. Live cells were stained for surface antigens immediately after dissociation. A pellet of 10^6 cells was resuspended in $50 \mu\text{l}$ of MP (1 mg ml^{-1} in PBS) for 20 min at 4°C . Cells were then washed twice, incubated with FITC GaM α (0.5 mg ml^{-1}), washed again, resuspended in a minimal volume and sealed with a coverslip on a microscope slide.

On mature cultures, all cells with neuronal morphology (that is, phase bright somata, distinct nuclei and process extensions¹) were stained with PC-BMP MOPC 167 whereas flat background cells (presumably fibroblasts and glial cells) were non-reactive (Fig. 1). A cell type indistinguishable from that stained by MOPC 167 was also stained by antiserum to NSE (a cytoplasmic marker) (Fig. 2) and by tetanus toxin. The latter was used in a double-labelling experiment and all the cells which showed positive fluorescein staining by PC-BMP and FITC GaM α were also stained by tetanus toxin conjugated to rhodamine. However, labelled tetanus toxin also stained some background cells. Identical results were obtained when PC-BMPs other than MOPC 167 were used. Thus, TEPC 15 and McPC 603 behaved exactly like MOPC 167. MP McPC 870 also stained neurones in the same way, although it binds PC with much lower affinity than do the other PC-BMPs¹⁰. The binding of PC-BMPs to neurones

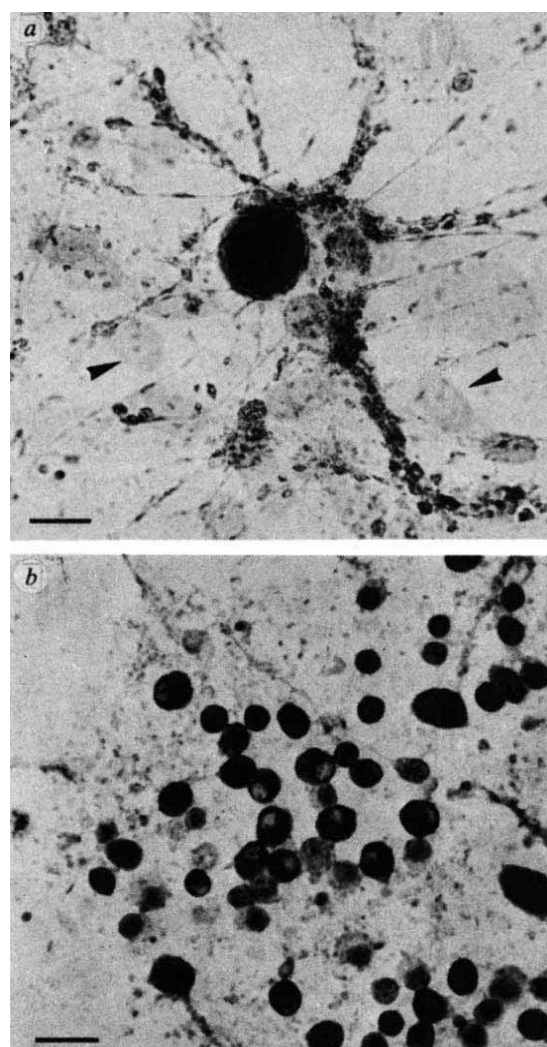


Fig. 2 Immunoperoxidase staining of mouse spinal cord and dorsal root ganglia cells after 4 weeks in culture. Cells were fixed in 4% paraformaldehyde, 1% glutaraldehyde, 0.2% picric acid and 1.5% sucrose in 0.1 M sodium acetate buffer, pH 6.0, for 1 h. The fixed cells were washed in 0.05 M Tris-HCl and 0.15 M NaCl, pH 6.0, then treated with 0.25% Triton X-100 in the same buffer for 10 min, and washed again. The monolayers were incubated with rabbit antiserum to neurone-specific enolase (1:800 in PBS) then with protein A-peroxidase (0.05 mg ml^{-1}) and finally reacted with diaminobenzidine and H_2O_2 , as previously described⁷. Monolayers were counterstained with cresyl violet, dehydrated and mounted. Neurones show positive staining for NSE in their soma as well as their processes. In addition to the labelled neurones, the nuclei of several unlabelled cells can be seen (arrowheads). Scale bars, $33 \mu\text{m}$ (*a*) and $40 \mu\text{m}$ (*b*).

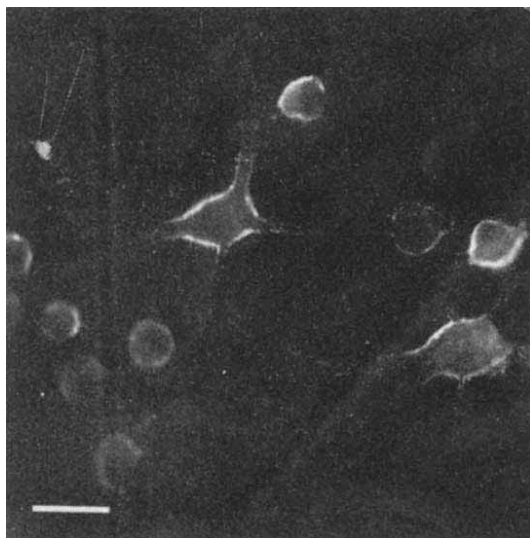


Fig. 3 Immunofluorescence of unfixed mouse spinal cord and dorsal root ganglia cells. After 4 weeks in culture, cells were stained as in Fig. 1 but without fixation. The staining pattern is typical of membrane antigens. Scale bar, 40 μ m.

was completely inhibited by a preincubation of the proteins with 10^{-2} M PC, suggesting that PC is part of the determinant. Other MPs of the IgA class (XRPC 24, XRPC 25, UPC 61, EPC 109) did not bind to any cell type in our cultures. MOPC 167 also did not bind the fibroblastic lines, 3T3 4E (ref. 17), cl ID (ref. 18); A9 (ref. 19) and DVGK (a kidney fibroblast-like cell line); a mouse liver cell line, ML311 (established by H. Coon); and a neuroblastoma cell line, N4TG1 (ref. 20).

The agreement between our observations and those obtained with accepted markers for neurones indicates that the receptor for PC-BMPs can serve as a marker for neurones in cultured murine nerve cells. It is not yet known whether the antigen is neurone-specific or simply more exposed than on other cell types. An important advantage of our method is that it can be used on live cells (Fig. 3). In addition, with our reagents the labelling was stronger and more specific than labelling with tetanus toxin, which also stained some background cells. It was surprising that although PC is present on the surface of most eukaryotic cells, PC-BMPs reacted only with neurones. Regardless of the reason for this binding, the observation that live cells could also be stained by this method offers the possibility of both identifying and separating neurones from mixed nerve cell populations using methods such as the fluorescence-activated cell sorter²¹.

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Morphological and biochemical maturation of neurones cultured in the absence of glial cells

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Cultures of isolated neurones offer possibilities for investigating their properties directly in the absence of glial cells. Furthermore, such a culture provides a suitable model for the study of the biochemical and functional maturation of neuronal cells in a well defined molecular environment. Culture techniques of isolated neurones have been developed for cells originating from the peripheral nervous system¹, especially sympathetic ganglion cells^{2–5}. Neurones from the central nervous system (CNS) have proved more difficult to maintain in culture in the absence of other cell types⁶. It is only recently that hippocampal neurones from rat embryos have been successfully maintained in culture⁷. We now describe a method for obtaining a pure culture of dispersed neurones from the telencephalon of 8-day-old chick embryos. This method is derived from a technique previously developed in our laboratory⁸. In those culture conditions, the neuronal cells were obtained by dissociation of cerebral hemispheres from 5–7-day-old chick embryos. The cells remained isolated for only 48 h after plating. Beginning with the 3rd day *in vitro*, the cells clumped together and formed large aggregates in which it was quite difficult to identify the cell types. We have improved those culture conditions and have succeeded in cultivating neurones which remained well dispersed for the whole period of culture.

We established the purity of the neuronal preparation by the morphology and by the binding of tetanus toxin, which is the most specific histological marker for neurones in culture⁹. We studied the maturational pattern of these cultures both morphologically and biochemically.

The two main improvements made to obtain cultures of pure dispersed neurones were the use of older embryos, 8–11 days old, and the technique of preparation of the polylysine substrate. Polylysine-coated surfaces were first developed by Yavin¹⁰ and by Letourneau¹¹ to improve the attachment of the cells to the tissue culture dish. The polylysine solution was prepared immediately before use by dissolving 10 mg of polylysine (Sigma, type IB or VIIB, PM 70,000) in 1 l of a 0.1 M boric acid–NaOH buffer, pH 8.4. After sterilisation by Millipore filtration, 10 ml of the polylysine solution were added to each Petri dish (Falcon, 100 mm diameter) for 24 h at room temperature. After removal of the polylysine solution, the plates were rinsed once with 10 ml of Dulbecco's modified Eagle's medium (DMEM, GIBCO), filled with 6 ml of nutrient medium (80% DMEM + 20% fetal calf serum) and placed in a CO₂ incubator (37°C, 95% air–5% CO₂). The cell suspension was prepared from the telencephalon of 8–11-day-old chick embryos as previously described⁸. The final concentration of the cell suspension was adjusted to 10⁶ cells per ml of nutrient medium. Of this suspension, 4 ml, corresponding to one cerebral hemisphere, were added to each prepared dish and the cultures were immediately placed in the incubator. After 3 days incubation the medium was removed and replaced by fresh medium. The

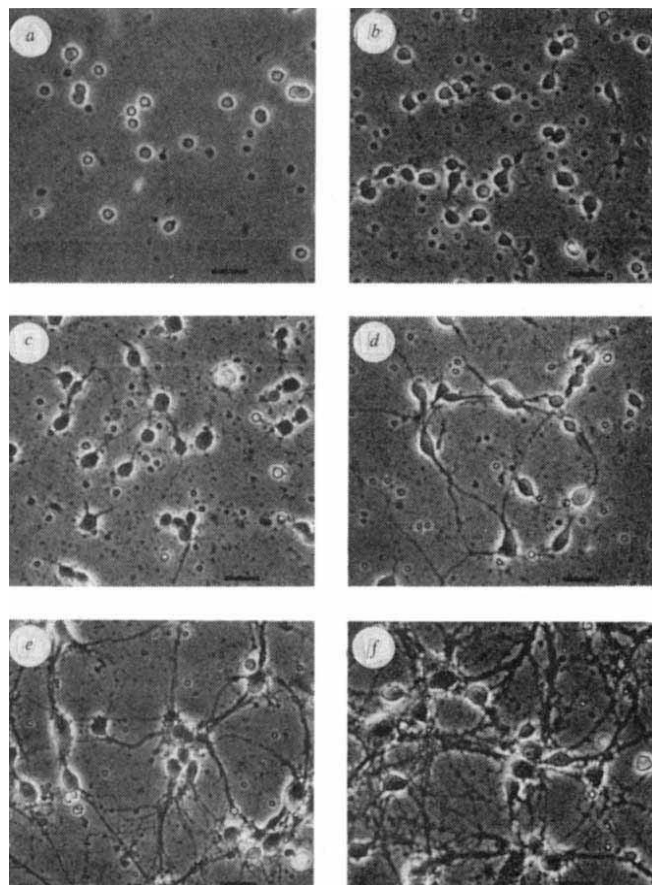


Fig. 1 Morphological development of cultured brain cells dissociated from 8-day-old chick embryos and grown on polylysine-coated Petri dishes. Observations by phase-contrast microscopy. Each bar represents 30 μm . a, Initial cell suspension; b, 12 h; c, 24 h; d, 2 days; e, 4 days; f, 7 days in culture. Detailed culture conditions are described in the text.

cultures remained healthy until the 10th day, as seen by trypan blue coloration.

Figure 1a shows that the dissociation of the telencephalon into single cells was complete. The cells adhered very rapidly (within 2–5 min) to the polylysine substrate. After 6 h of incubation, the plating efficiency of the initial suspension was $98 \pm 0.5\%$. The further morphological development of a typical culture is shown in Fig. 1b–f. The criteria used for the evaluation of the morphological maturation were the size of the cell bodies and the length and complexity of the processes. The outgrowth of processes began between 6 and 12 h (Fig. 1b). However, at 12 h, many of the cells showed no indication of neurite formation. Between days 1 and 7, the size of the cell bodies and the number of fibre-bearing cells increased, and the processes spread out and became progressively more ramified, until they formed a dense network covering the entire plate (Fig. 1c–f).

The most important aspect of our work is the obtention of pure neuronal cultures, as 98% of the cells bind the tetanus toxin (Fig. 2a). The absence of glial cells in our cultures could be due partly to the fact that gliogenesis is known to occur later than 8 days during the *in vivo* development of the chick embryo, and partly to the fact that the proliferation in culture of the few glial cells present in the initial cell suspension is inhibited by the polylysine substrate. Non-neuronal proliferation is further prevented by the complete absence of clump formation in our cultures. To estimate the number of glioblasts remaining in the neuronal cultures the cells from a 7-day-old culture were carefully scraped from their dishes, rinsed with serum-free medium, centrifuged at 2,000 r.p.m. for 5 min and transferred to a collagen-coated Petri dish. This substrate is known to favour

glial proliferation and maturation. The cells were allowed to grow for 24 h in DMEM containing 20% fetal calf serum and the glial cells present and spread out in the dish were counted under phase-contrast microscopy. The number of glial cells corresponded to less than 0.1% of the total cell population. In any event, our cultures show striking differences from most of those previously described for CNS neurones of embryonic chick or rat (ref. 7 excepted), in which the proliferation of glial cells cannot be entirely inhibited, even in the presence of antimetabolic drugs^{12,13}.

We have correlated the morphological observations with the development of some cellular parameters (Fig. 3). Between the 1st and 3rd day, there is a doubling of the cell number per culture dish and a parallel doubling of the amount of DNA (Fig. 3a,b). The first change of medium on the 3rd day causes a decrease in the DNA content and cell number. After 4 days these two parameters remain constant. The amount of RNA per plate (Fig. 3c) increases until around the 6th day; the amount of protein

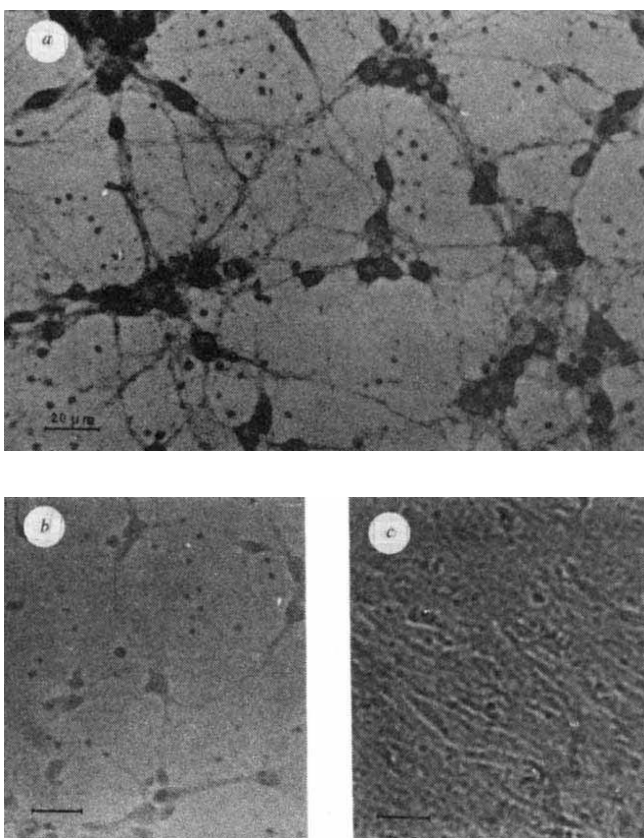


Fig. 2 Immunohistochemical demonstration of tetanus toxin binding sites in the neuronal cultures. a, 8-day-old chick embryo cerebral hemisphere neurones cultured for 5 days on polylysine-coated Petri dishes; stained with tetanus toxin, human anti-toxoid gammaglobulins and anti-human gammaglobulins coupled to peroxidase. b, 8-day-old chick embryos cerebral hemisphere neurones cultured for 5 days on polylysine-coated Petri dishes; control preparation, stained with tetanus toxin and with anti-human gammaglobulins coupled to peroxidase but without toxoid antiserum. c, 20-day-old glial cell cultures, from 15-day-old chick embryo cerebral hemispheres, stained as in a. The reaction was always negative in these cultures. Each bar represents 20 μm . The immunohistochemical reaction for the localisation of tetanus toxin binding sites was carried out as described by Mirsky *et al.*⁹. The cultures were rinsed three times with a 0.1 M phosphate buffer, pH 7.4, and fixed with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 20 min. After washing, the cells were incubated with 200 μl of tetanus toxin solution ($10 \mu\text{g ml}^{-1}$ in DMEM), then with either human gammaglobulins or rabbit anti-horse gammaglobulins conjugated with fluorescein isothiocyanate (Nordic Limited). For each step, the cells were incubated for 30 min at room temperature. Peroxidase activity was revealed by a 0.05% (w/v) solution of diaminobenzidine in 0.1 M phosphate buffer, pH 7.4, containing 0.5% (v/v) H_2O_2 . In the control preparations, either the tetanus toxin or its antiserum was omitted.

(Fig. 3d) increases until the 8th day. These increases occurring after cell proliferation has ceased may be related to the observed increases in the size of the cell bodies and the extension of the processes. Tyrosine hydroxylase activity (Fig. 4) was present in our cultures and showed an eightfold increase between the 1st and 7th days of culture. Dopamine- β -hydroxylase activity was not detected. These morphological and biochemical results indicate the existence of a maturation process, despite the short life span of the cultures.

To our knowledge, as far as the high plating efficiency, the survival of the cells and the purity of the neuronal preparation are concerned, no comparable observations have been reported for central neurones in culture. This culture system was developed with cells originating from the whole telencephalon, but we also obtained pure cultures of neurones from more specific regions of the brain from chick embryos such as striatum, cortex, thalamus, pons-medulla and the optic lobes. Such a culture system will be useful for the study of biochemical and possibly functional maturation of neuronal cells. It will also

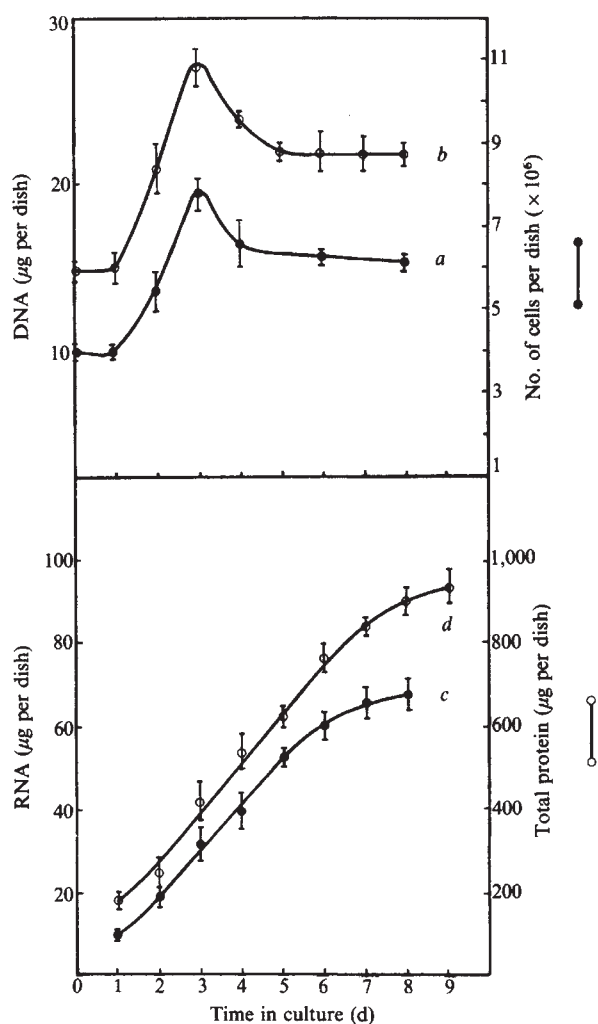


Fig. 3 Biochemical development of isolated neurones in culture. *a*, Number of cells per dish (●). The cells were collected in 10 ml of 0.9% NaCl and aliquots were counted in a Neubauer haemocytometer. Each point represents the mean count $\pm$ s.d. of triplicate determinations of three cultures each. *b*, *c*, *d*, Amounts of DNA (○), RNA (●) and total protein (○) per plate, respectively. The cultures were rinsed three times with a 0.9% NaCl solution, collected into 5 ml of 0.9% NaCl and centrifuged at 3,000 r.p.m. for 5 min. The pellet was sonicated twice for $\times 20$ s with a 20-s interval in 1 ml of 2 M NaCl solution containing 5 mM EDTA and 50 mM Tris-HCl (pH 8). DNA was determined by the method of Burton¹⁴, RNA by the method of Meijbaum¹⁵ and total proteins by the method of Lowry¹⁶. Each point represents the mean value $\pm$ s.d. of triplicate determinations of six cultures each.

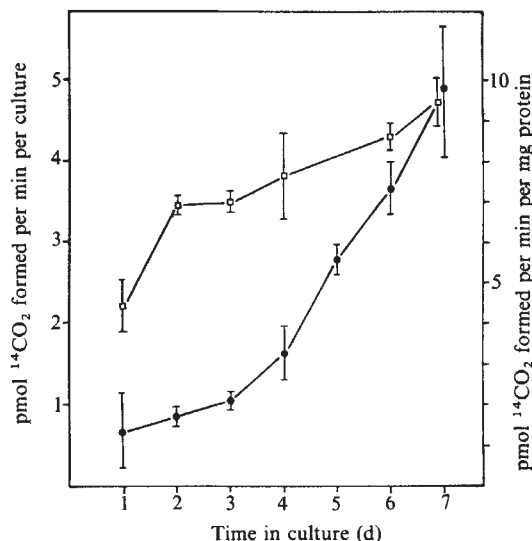


Fig. 4 Developmental pattern of tyrosine hydroxylase (TH) activity in cultures of 8-day-old chick embryo cerebral hemispheres, grown on polylysine, expressed per mg protein (□) and per culture (●). For the determination of TH activity, the cultures were collected in 50 μ l of 0.05 M Tris, pH 6.0 (acetate), containing 0.2% Triton X-100, homogenised in glass microhomogenisers and centrifuged for 15 min at 12,000g. TH activity was assayed on 10 μ l (corresponding to 15–40 μ g protein) of this supernatant, by the $^{14}\text{CO}_2$ -trapping method described by Waymire *et al.*¹⁷, with slight modifications. The final concentrations of the incubation mixture were as follows: 0.17 M sodium phosphate buffer (pH 6.2), 0.8 mM FeSO_4 , 0.3 mM pyridoxal phosphate, 30 units ml^{-1} catalase (Boehringer), 0.2 units μl^{-1} dopa decarboxylase (1 unit = 1 nmol $^{14}\text{CO}_2$ formed per min) prepared from pig kidney¹⁷, 8.0 mM DMPH₄ (Aldrich), 30 mM 2-mercaptoethanol and 150 μM L-[1- ^{14}C]-tyrosine (specific activity: 50 mCi mmol^{-1} ; Amersham). The reaction was carried out for 20 min at 37°C in tubes sealed with rubber injection vial caps. A plastic well was suspended from the rubber injection stopper, which contained 100 μ l Protosol (Packard) to trap $^{14}\text{CO}_2$ formed during the assay. The reaction was stopped by injecting 100 μ l of 3M H_2SO_4 and the tubes were replaced for an additional period of 20 min at 37°C. The Protosol was transferred to counting vials containing 10 ml of Monophase 40 scintillation liquid (Packard). The counting efficiency was 95%. Each point represents the mean value $\pm$ s.d. of 8–10 cultures from 3 different experiments. For the determinations of dopamine- β -hydroxylase activity, the cells were homogenised in 5 mM Tris-HCl buffer (pH 6.5) containing 0.1% Triton X-100 and the homogenate was assayed according to Bouclier *et al.*¹⁸.

allow an extensive characterisation of pure neurones, for in the present conditions the neuronal population is not contaminated by glial cells, as is the case with bulk-prepared neurones.

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Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: evidence for a pre-CFU-S cell?

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The spleen colony assay of Till and McCulloch¹, which is a clonal assay², is considered to be a measure of the stem cell content of haematopoietic tissue. Stem cells are a heterogeneous population³⁻⁶ with respect to their capacity to generate further stem cells (assayed as colony-forming units in spleen, CFU-S) and progenitors of red cells, granulocytes, macrophages and platelets. It has been suggested that stem cells are organised for use according to their generation age^{5,6}. There is evidence to indicate that drugs such as hydroxyurea (HU) and 5-fluorouracil (5-FU), which preferentially eliminate proliferating cells, spare non-cycling stem cells which have a high capacity to generate other stem cells⁶ and progenitors of granulocytes and macrophages⁶ as well as megakaryocytes⁷. We show here, however, that the CFU-S content of haematopoietic tissue is not an adequate predictor of the capacity of that tissue to repopulate bone marrow of a lethally irradiated host. Furthermore it appears that haematopoietic stem cells are not only heterogeneous with respect to their capacity to generate other stem cells and progenitors of haemic cells in the spleen but also differ in their capacity to 'home' to either the bone marrow or spleen. The evidence suggests a class of 'primitive' haematopoietic stem cell (pre-CFU-S) that accumulates in the bone marrow to give rise to cells capable of growing in spleen.

During studies of the properties of the haematopoietic stem cells that survive 5-FU treatment we observed that the number of colonies per spleen, in irradiated mice which had been injected with bone marrow from 5-FU treated donors (FUBM), showed a marked increase between 8 and 14 days after irradiation (Fig. 1a). The spleen weights of irradiated mice injected with FUBM also showed a remarkable increase between days 12 and 14 (Fig. 1b). In contrast, spleen colony counts remained constant as had been previously noted⁸, and spleen weights only increased slightly, in mice injected with normal bone marrow (NBM).

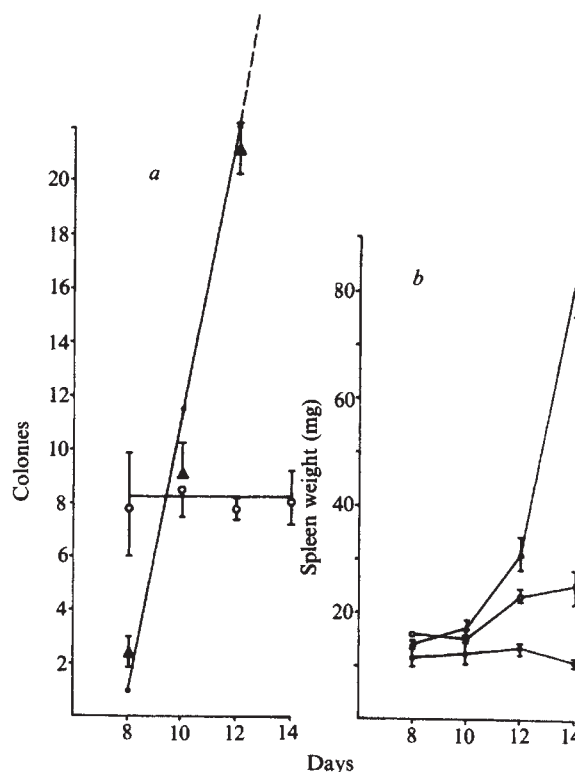


Fig. 1 a, Colony counts plotted against time. Bone marrow donors were 10 normal 10–12-week-old (BALB/c × C57BL/6)F₁ male mice or 10 F₁ mice which had been injected intravenously 4 days previously with 5-FU 150 mg per kg. Groups of 10 850R X-ray irradiated F₁ mice, injected with either normal BM, FUBM or uninjected controls were anaesthetised with ether and then killed by cervical dislocation at 8, 10, 12 or 14 days after irradiation, the spleens were removed and fixed in FAA (formalin/acetic acid/alcohol solution) for 24 h then formalin pH 7.4. Colonies were counted (using a dissecting microscope ×16.5 magnification). The linear correlation coefficient *r* was 0.8937 for colony counts against time, for FUBM injected mice and 0.1201 for NBM-treated mice. The slope (colonies per day) was 5.33 ± 0.62 for FUBM treated mice and not significantly different from zero for NBM treated mice. b, Weights of fixed spleens of mice injected with normal bone marrow or with bone marrow removed 4 days after 5-FU injection. ▲, FUBM injected mice; ○, NBM injected mice; ●, X-ray irradiation controls.

In other experiments using marrow of hydroxyurea-treated donors whose CFU-S have a higher than normal CFU-S generating capacity⁶, the phenomenon of time dependence of spleen colony counts was also observed although to a lesser

Table 1 CFU-S¹ and GM-CFC⁹ content and marrow (MRA) and spleen (SRA) repopulating ability of femurs and spleen of X-ray irradiated female (BALB/c × C57BL/6)F₁ mice injected with either normal bone marrow (NBM) or marrow obtained from donors injected with 5-FU 1 day previously (FUBM)

Donors	CFU-S dose	Spleen			Femur			SRA per femur
		Colonies	CFU-S × 10 ⁻³	GM-CFC × 10 ⁻³	CFU-S × 10 ⁻³	GM-CFC × 10 ⁻³	MRA × 10 ⁻³ per femur	
FU-BM 0.2 femur	1.7 ± .4	13.1 ± 1.2	1.2 ± 1.12	137 ± 13	0.72 ± 0.10	52.8 ± 4.2	42.4 ± 7.6	200 ± 40
NBM 0.025 femur	194* ± 11	Confluent	4.03 ± 0.40	177 ± 6	0.190 ± 0.016	7.8 ± 1.3	2.8 ± 0.2	64 ± 13
Radiation control	0	0	ND	0	ND	0†	0†	0†

* The dose of CFU-S in NBM is estimated from a colony count made on day 10 of spleens obtained from irradiated mice injected with 0.0025 femur obtained from normal mice. GM-CFC content was calculated from the mean of five replicate dishes plated with an appropriate aliquot of a pooled femoral marrow cell suspension from 10 mice. † No colonies were seen in five dishes plated with 1/100 of a femur of marrow from irradiation control mice. ND, not determined.

degree ($r = +0.7961$; slope 3.2 colonies per day) than in the 5-FUBM experiment.

Our results indicate that estimates of CFU-S content of marrow suspensions based on a 10-day spleen colony count are not meaningful where the colony count is varying with time. It is as if the haematopoietic stem cells which survive 5-FU preferentially accumulate in the marrow where they give rise to large numbers of CFU-S, some of which then migrate to the spleen where they give rise to delayed colony development.

In relation to bone marrow-seeking stem cells we have repeatedly observed (see, for example, Table 1) that irradiated mice injected with FUBM (from mice 1 to 4 days after FU treatment), had few (13–22) colonies in the spleen but had a higher content of GM-CFC and CFU-S in their femurs at day 13 than mice injected with suspensions of NBM containing about 200 CFU-S. The interpretation of the assay for CFU-S content of cell suspensions obtained from irradiated mice injected with FUBM was not complicated by a change of spleen colony counts in the secondary irradiated recipients between days 10–14. Marrow from mice injected with a bone marrow suspension taken 1 day after 5-FU injection, which produced only 1.7 spleen colonies at day 10, and 13 at day 13, contained four times more CFU-S and six times more GM-CFC than that of mice injected with a dose of normal bone marrow containing 197 CFU-S (Table 1). The ratio of the CFU-S content of spleen to the colony count at day 13 was 100:1 for mice injected with FUBM and 20:1 in mice injected with NBM (calculated value based on CFU-S dose). The ratio GM-CFC: average spleen colony was 10,000:1 and 1,000:1 for FUBM and NBM respectively.

The marrow (MRA) and spleen (SRA) repopulating ability of marrow cell suspensions prepared from X-ray irradiated recipients injected with either NBM (X+NBM) or FUBM (X+FUBM) was evaluated by injection of cell suspensions into secondary irradiated recipients and measuring the GM-CFC content of femurs and spleen on day 13. The results (Table 1) are expressed as GM-CFC per organ per femur injected. Mice injected with marrow from X+FUBM had ~14 times more GM-CFC in femora and 3 times more in spleen than those injected X+NBM marrow. If one takes 1 femur = 1/20 of total marrow, it can be seen that X+FUBM marrow generates many more GM-CFC in marrow than in spleen, while that from NBM marrow generates as many spleen and bone marrow GM-CFC in secondary X-ray irradiated recipients. Results of primary and secondary transplants show that 'stem' cells, efficient in marrow repopulation are affected minimally, if at all, by a dose of 5-FU that reduces cells capable of forming colonies in spleen at day 10 ('10 day CFU-S'), to about one-thousandth of that of normal.

The non-cycling marrow repopulating stem cells which survive 5-FU treatment are associated with a special class of primitive progenitors, which can form very large colonies (containing 5×10^4 cells) of macrophages in agar after 10 days of culture¹⁰. These progenitors may prove to be the best 'in vitro' indicator of the marrow repopulating ability of suspensions of haematopoietic cells.

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Erythropoietin-stimulated erythropoiesis in long-term bone marrow culture

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The proliferation of multipotential haematopoietic stem cells (CFU-S) is possible in some long-term bone marrow cultures^{1–3}. Granulocyte and macrophage progenitors (CFU-C)² and megakaryocyte precursor cells (CFU-M)⁴ are present in these cultures and undergo full development into mature cells^{5–7}. In contrast, while immature erythroid progenitors ('early' BFU-E) are maintained in long-term culture, none of the more differentiated progeny (CFU-E) have been detected, and no morphologically recognisable erythroid cells have been observed^{8–11}. We now describe a modified culture system in which the 'early' BFU-E develop into 'late' BFU-E in response to added erythropoietin. Further maturation of these cells into CFU-E and non-nucleated erythrocytes can be achieved by mechanical agitation of the long-term cultures or by transferring the cells into dishes which do not allow cell attachment to occur.

One reason for our previous failure to detect erythroid development in erythropoietin-stimulated long-term marrow cultures^{8,9} could be the presence of horse serum in the growth medium used to establish the cultures, which may be inhibitory for erythroid colony growth¹¹. On the other hand, fetal calf serum (FCS), which is usually used for the clonal assay of erythroid progenitor cells in semi-solid media, is defective in its ability to stimulate the formation of a competent haematopoietic inductive micro-environment *in vitro* (including endothelial cells, fat accumulating cells and macrophages)³ and therefore in its ability to sustain *in vitro* haematopoiesis. We have taken advantage of the recent finding that the addition of hydrocortisone to FCS will support maintenance and proliferation of CFU-S and CFU-C in culture (perhaps by influencing the formation of fat accumulating cells in the adherent layer)¹².

The lack of erythropoiesis in long-term marrow cultures may also be related to the finding that the progression from 'early' BFU-E to haemoglobin synthesising cells in erythropoietin-stimulated cultures may require some form of semi-solid support (such as methylcellulose) in the medium¹³. Perhaps the role of methylcellulose in these cultures is to prevent the cells from settling and subsequently attaching to the bottom of the culture dishes. We have used two different approaches to prevent cell adherence in these experiments. The first method was to transfer the cells from the long-term marrow cultures to plastic Petri dishes designed for bacterial culture. Preliminary investigation had indicated that a much smaller proportion of the long-term culture cells will attach to these dishes as compared with standard tissue culture dishes. Our second approach was to place the established long-term cultures on a mechanical shaker to prevent attachment of the erythroid progenitor cells by physical means.

The long-term cultures were established with pooled femoral marrow cells (from 10-week-old male B6D2F₁ mice) suspended at a concentration of 1.3×10^6 cells per ml in alpha medium with nucleosides (GIBCO Bio-Cult) supplemented with 25% FCS (Flow), 1% bovine serum albumin (BSA; Path-o-cyte 7, Miles), 10^{-4} M 2-mercaptoethanol (BDH), 7×10^{-6} M human transferrin (Sigma) fully saturated with iron, 10^{-7} M sodium selenite and 10^{-6} M hydrocortisone-21-sodium succinate (Sigma). Aliquots of 10 ml of cell suspension were put into tissue culture flasks (Sterlin), gassed with 5% CO₂ in air and incubated at 33 °C. The cultures were 'fed' at weekly intervals by removing

Table 1 Effect of erythropoietin on erythroid precursor populations in long-term marrow cultures

Week	Group	Erythropoietin	Cells per ml $\times 10^5$	Colonies per 10^5 cells				
				CFU-E		BFU-E		
				day 2	day 4	day 4	day 10	day 14
2	A	—	4.2	ND	ND	ND	10 $\pm$ 3	22 $\pm$ 2
	B	—	4.0	ND	ND	ND	20 $\pm$ 3	45 $\pm$ 2
	C+D	—	4.0	ND	ND	ND	13 $\pm$ 2	28 $\pm$ 9
	E	—	3.8	ND	ND	ND	13 $\pm$ 3	38 $\pm$ 4
3	A	—	4.4	0	0	1 $\pm$ 1	ND	ND
	B	+	6.4	1 $\pm$ 1	2 $\pm$ 1	12 $\pm$ 1	ND	ND
	C	—	20.2	0.2 $\pm$ 0.2	0	0.9 $\pm$ 0.4	ND	ND
	D	+	25.0	76 $\pm$ 2	ND	9 $\pm$ 1	ND	ND
	E	+	7.6	69 $\pm$ 4	ND	16 $\pm$ 4	ND	ND
4	A	—	2.2	0	0	0	7 $\pm$ 4	7 $\pm$ 1
	B	+	2.0	0	6 $\pm$ 2	10 $\pm$ 5	5 $\pm$ 4	5 $\pm$ 4
	C	—	1.7	0	0	0	0	0
	D	+	11.0	41 $\pm$ 4	5 $\pm$ 3	3 $\pm$ 0	0	0
	E	+	2.1	11 $\pm$ 2	0	0	0	0

Long-term culture groups: A, control; B, erythropoietin at week 2; C, adherent and suspension cells transferred to Petri dishes at week 2 without erythropoietin; D, same as C with erythropoietin; E, suspension cells transferred to Petri dishes at week 2. For erythroid colony assays, cells were cultured at $1\text{--}2 \times 10^5$ cells ml^{-1} in alpha medium supplemented with 0.9% methylcellulose, 16% FCS, 0.9% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, 7×10^{-6} M transferrin, 10^{-7} M Na_2SeO_3 and 3×10^{-5} M egg lecithin. Four 0.25-ml aliquots of each cell suspension were plated in the wells of Costar (FB-16-24-TC) tissue culture clusters and incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. Colonies derived from day 2 and day 4 CFU-E (1 or 2 clusters of haemoglobin containing cells) and day 4 BFU-E (3 or more clusters) were grown in the presence of 0.5 U ml^{-1} sheep plasma erythropoietin (step III; Connaught). The assay cultures for day 10 and day 14 BFU-E contained 1.8 U ml^{-1} step III erythropoietin and burst feeder activity as supplied by 1×10^6 irradiated cells (15 Gy from a ^{137}Co source)¹⁵. The results are expressed as the mean $\pm$ 1 s.d. ND, not determined.

half the growth medium and replacing it with fresh medium. After 2 weeks of incubation (to allow an adherent layer to develop), 15×10^6 freshly isolated, syngeneic bone marrow cells were added. This time was taken as week zero. At week 2, duplicate cultures were treated according to the following protocols: A, control long-term cultures; B, erythropoietin-stimulated long-term cultures; C, suspension and adherent cells replated in bacterial Petri dishes; D, replated suspension and adherent cells with erythropoietin; and E, replated suspension cells alone with erythropoietin. The control cultures (A) were fed in the usual manner with no further additions. Erythropoietin-stimulated cultures (B) were given 10 units of highly purified human urinary erythropoietin which was a hydroxylapatite fraction with a specific activity of 70,000 U per mg of protein. The level of erythropoietin was maintained at 1 U ml^{-1} through week 4 by further addition of erythropoietin with each medium change. Groups C and D were set up by removing half the suspension cells in growth medium and all of the adherent cells from a third set of long-term cultures (C+D) and mixing them with an equal volume of fresh medium. Aliquots of 5 ml were plated in 60-mm diameter bacterial Petri dishes (Sterilin) without (C) and with (D) 1 U ml^{-1} highly purified erythropoietin. The final group (E) contained suspension cells alone from the 2-week-old long-term cultures, diluted with an equal volume of fresh medium, and plated in Petri dishes with 1 U ml^{-1} erythropoietin. The secondary cultures (C-E) were

re-fed after 1 week by removing half the medium and replacing it with fresh medium. Pooled suspension cells from the long-term cultures were assayed before treatment at week 2, and after treatment at weeks 3 and 4, for erythroid progenitor cells in a modification of the methylcellulose system originally described by Guilbert and Iscove^{14,15}.

The results of the erythroid colony assays are given in Table 1 and the morphological composition of the cultures at week 3 is shown in Table 2. The 'early' BFU-E (colonies scored at day 10 or day 14) are present in the erythropoietin-treated long-term cultures (B) up to week 4 at levels comparable to those found in the control cultures (A), as are the numbers of CFU-S (data not shown). The erythropoietin-treated cultures also had significant numbers of intermediate erythroid progenitors, 'late' (day 4) BFU-E and day 4 CFU-E, at weeks 3 and 4, whereas there were essentially none in the control cultures at these times. However, very few day 2 CFU-E or morphologically recognisable erythroid cells were detected in the intact erythropoietin-stimulated long-term cultures. In contrast, the erythropoietin-treated, replated cultures (D and E) contained many day 2 CFU-E and benzidine-positive erythroid cells including non-nucleated cells were present at week 3.

Since erythropoietin stimulated the formation of haemoglobin-containing cells when long-term culture cells were transferred to bacterial Petri dishes, it was of interest to find whether erythroid maturation could occur in the intact long-term

Table 2 Morphology of suspension cells from erythropoietin-treated long-term cultures at week 3

Group*	Erythropoietin	Morphology %				Benzidine-positive non-nucleated cells
		Blasts	Granulocytes	Mononuclear cells	Erythroid cells	
A	—	0	75	25	0	—
B	+	1	96	3	0	—
C	—	1	98	1	0	—
D	+	1	69	2	28	+
E	+	2	82	5	12	+

*As detailed in Table 1.

Table 3 Effect of mechanical agitation on erythropoietin-stimulated erythropoiesis in long-term marrow cultures

Week	Erythropoietin units ml ⁻¹	× 10 ⁵ cells per ml	Erythroid colonies per 10 ⁵ cells		% Morphology				Benzidine- positive non-nucleated cells
			day 2	day 4	Blasts	Granulocytes	Mononuclear cells	Erythroid cells	
5	0	8.0	0	0	3	97	0	0	—
	1	12.6	4 ± 3	13 ± 1	0	93	7	0	—
6	0	17.2	0	0	0	95	4	0	—
	0.5	24.0	20 ± 5	15 ± 2	0	64	6	30	+

The cultures were established in Fischer's medium supplemented with 25% FCS and 10⁻⁶ M hydrocortisone. Four weeks after the second inoculum of bone marrow cells was added, 10 units of sheep plasma erythropoietin (step III) were added to two of the four 10-ml cultures which were transferred to a 37 °C incubator and were placed on a tilting platform shaker (Luckham Ltd). The clonal assays for erythroid progenitor cells were performed as described in Table 1 legend.

cultures if they were mechanically agitated. The results of the shaker experiment are shown in Table 3. After 1 week (week 5) the erythropoietin-treated cultures had high numbers of progenitors capable of giving rise to erythroid colonies at day 4, but no morphologically recognisable erythroid cells were detected in the long-term cultures at this time. On the other hand, by week 6, appreciable numbers of day 2 CFU-E were present in the treated cultures and there were also significant numbers of benzidine positive-erythroid cells. The apparent delayed development of erythroid cells in this experiment, after 2 weeks incubation as compared with only 1 week in the previous experiment (Table 1), is probably due to the impure sheep plasma erythropoietin used in this experiment. This preparation of step III erythropoietin (lot no. 3023-1) may be toxic to the late erythroid progenitors since there are decreased numbers of day 2 erythroid colonies formed by freshly plated marrow cells at erythropoietin concentrations above 0.5 U ml⁻¹ in the methylcellulose assay system (data not shown).

Thus we have shown that the addition of erythropoietin to long-term cultures will induce the production of intermediate erythroid progenitors, day 4 BFU-E and CFU-E. However, erythropoiesis seems to be blocked at this stage of maturation because no day 2 CFU-E or morphologically recognisable erythroid cells are found. Transfer of the long-term culture cells to an environment where cell adherence is inhibited allows further maturation of the cells, although there is a rapid decline in the precursor populations as demonstrated by the absence of day 10 and day 14 BFU-E at week 4 (groups C-E in Table 1). The latter effect is presumably due to the lack of an effective inductive environment (presumably supplied by the adherent layer in the long-term cultures). The presence of the adherent layer in the long-term cultures is not, of itself, inhibitory to erythroid development, as haemoglobin-containing cells are formed in erythropoietin-treated long-term cultures which have been incubated on a mechanical shaker.

We propose a mechanism for the block in erythroid maturation in the epo-stimulated long-term cultures which involves the erythroid precursors becoming adherent as they mature to the CFU-E stage. The potentially adhering property of late erythroid progenitors is supported by the finding that they are retained on nylon fibre columns¹⁶. The adherent nature of these cells may be involved in the formation of erythroid islands which can be found in bone marrow and other sites of active erythropoiesis^{17,18}. We propose that if, *in vitro*, these cells adhere to unfavourable surfaces (and the adherent layer in the long-term cultures of the tissue culture flask, may be such) further maturation will not occur. On the other hand, if they are prevented from attaching to such surfaces then erythropoiesis can continue.

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Vascular endothelial and smooth muscle cells in culture selectively release adenine nucleotides

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Endothelial cells in culture can modulate platelet aggregation and vascular tone, in part by producing prostacyclin (PGI₂)^{1,2}, a powerful vasodilator and inhibitor of platelet aggregation^{3,4}, but also by their ecto-ADPase activity⁵, which initiates the conversion of pro-aggregating ADP to adenosine, a potent vasodilator⁶ and platelet inhibitor⁷. We have now demonstrated that cultured aortic endothelial cells exposed to trypsin, thrombin or other stimuli can liberate a high proportion of their adenine nucleotides without substantial loss of lactate dehydrogenase. ADP rapidly accumulates extracellularly, reaching biologically active concentrations before there is further breakdown to adenosine. Whether this selective release of nucleotides is a response to damage, or whether it represents a specific secretory mechanism remains to be resolved. Cultured aortic smooth muscle cells can secrete adenine nucleotides in a similar manner, but extracellular conversion to adenosine occurs much faster.

The production of PGI₂ has previously been demonstrated by the ability of cultured vascular cells to inhibit platelet aggregation induced by a variety of stimuli^{1,2,8-10}. Using porcine aortic

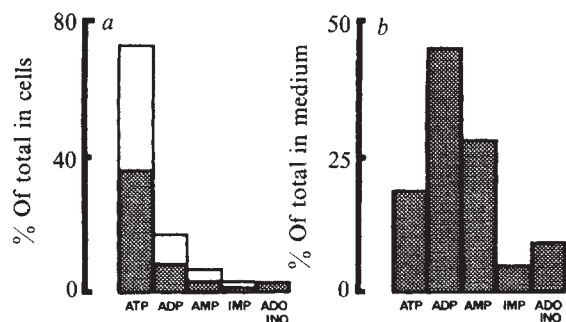


Fig. 1 Adenine nucleotides of cultured porcine aortic endothelial cells were labelled by incubating replicate confluent monolayers in 16-mm diameter wells ($\approx 10^5$ cells per sample) for 60 min with $2 \mu\text{M}$ ^3H -adenosine¹¹. The cells were rapidly rinsed three times, then samples were treated with 0.1% trypsin plus 0.025% EDTA in PBS at 37 °C for 5 min, and the supernatant was separated from suspended cells by rapid centrifugation. Purines extracted from the cells before or after treatment with trypsin plus EDTA, or in the supernatant after treatment, were separated by thin layer chromatography and the radioactivity in each fraction was determined¹¹. Radioactivity associated with inosine was usually only $\sim 5\%$ of the total, and adenosine and inosine have been combined here (ADO, adenosine; INO, inosine). In *a*, the total height of each column represents the percentage of the total radioactivity incorporated by the cells into each compound. The shaded portion of each column represents the percentage (again of the original radioactivity) in each compound after treatment with trypsin plus EDTA. Thus, the unshaded portion of each column shows the labelled compounds lost from the cells. *b* Shows the distribution of extracellular radioactivity, expressed as percentage of the total release.

endothelial cells in culture¹¹, we showed that PGI_2 production was blocked by low concentrations of aspirin¹², and during further experiments with aspirin-treated cells we found that they could aggregate platelets directly when cell suspensions were prepared from monolayer cultures by a conventional procedure: treatment with 0.1% trypsin plus 0.025% EDTA for 5 min at 37 °C followed by the addition of 0.1 volume of serum to inactivate the trypsin. This aggregation was inhibited by 1–10 μM 2-azido-AMP, which is an antagonist of the ADP receptor on platelets¹³, and when cell-free supernatants were prepared by centrifuging, these contained a stable pro-aggregatory compound whose action was similarly blocked by 2-azido-AMP. The aggregation response was almost abolished when the supernatant was incubated for 50 min with 100 $\mu\text{g ml}^{-1}$ apyrase (Sigma, grade I). Treatment with 10 $\mu\text{g ml}^{-1}$ apyrase resulted in the response being initially potentiated ($>200\%$ of the control after 15 min) then reduced after longer incubation. This suggested that ATP and ADP were released from the endothelial cells by the procedure used to detach them; the ATPase activity of the apyrase was three to five times its ADPase activity (according to Sigma), and the results were therefore consistent with an initial conversion of ATP by apyrase and slower degradation of the ADP thus formed, together with that originally present.

To investigate the release of nucleotides in more detail, we took advantage of the fact that endothelial cells have a high-affinity transport process for adenosine through which ^3H -adenosine is predominantly incorporated into ATP and ADP within the cells¹¹. Cell suspensions were prepared from prelabelled monolayers by treatment with trypsin and EDTA as above; the cells were then separated from the extracellular medium by centrifuging, and the cellular and extracellular amounts and distribution of ^3H measured by thin layer chromatography and liquid scintillation counting¹¹. The fraction of the total cellular radioactivity released varied in different experiments (usually 10–30%, occasionally $>50\%$) but the pattern of ^3H release was highly consistent. In all experiments the main component lost from the cells was ^3H -ATP (Fig. 1*a*), but the largest fraction appearing extracellularly was ^3H -ADP (Fig. 1*b*).

Release of up to 60% of the intracellular ^3H , in a similar pattern, also occurred when cells were suspended by physical disturbance with a rubber policeman. To check that the radioactivity released did not represent a small pool of preferentially labelled nucleotides, we measured the total ATP content of cells and extracellular medium using luciferase¹⁴. The total cellular ATP concentration was $\sim 4 \text{ nmol per } 10^6 \text{ cells}$, of which up to 50% was released when cell suspensions were prepared using trypsin plus EDTA as described above.

The high fraction of cellular nucleotides released when the cells were suspended with trypsin plus EDTA was not accompanied by any evidence of cell damage as measured by vital dye exclusion (no detectable staining of cells with trypan blue) or cytoplasmic enzyme leakage (extracellular lactate dehydrogenase activity was $<5\%$ of the total cellular activity). Thus, the cells could liberate up to half their adenine nucleotides without the plasma membrane becoming significantly permeable to compounds of higher molecular weight. This selective release of nucleotides was not simply a consequence of the cells becoming detached from their substratum: there was always a small but significant release of nucleotides from prelabelled cells on replacing the medium above the monolayer with fresh medium, and this release was increased by adding agents such as collagenase or thrombin that did not detach the cell monolayer. For example, the addition of fresh culture medium alone at 37 °C for 5 min induced $7.2 \pm 1.4\%$ release of incorporated ^3H ; medium containing 0.2% collagenase released $14.0 \pm 0.7\%$ (mean $\pm$ s.d., $n = 4$). Treatment with 1–5 U ml^{-1} thrombin for 5 min at 37 °C released up to 25% of ^3H without visibly affecting cell morphology. The pattern of extracellular nucleotides found in these experiments was very similar to that obtained using trypsin plus EDTA. Nucleotide release was temperature dependent; for example, the addition of phosphate-buffered saline (PBS) alone for 5 min at 37 °C released $5.3 \pm 0.7\%$ whereas at 4 °C release was $2.3 \pm 0.7\%$ (mean $\pm$ s.d., $n = 4$). Release was not reduced when cells were treated with trypsin and EDTA in PBS containing 10 mM ATP (test release $26.5 \pm 3.0\%$; release in the absence of ATP $24.3 \pm 4.8\%$ (mean $\pm$ s.d., $n = 4$)). Also, there was no uptake of labelled exogenous nucleotide in conditions inducing selective release of intracellular nucleotides: when PBS containing 1 μM –4 mM ^3H -ATP ($\sim 2 \times 10^6 \text{ d.p.m.}$) plus 10–100 μM dipyridamole was added to an endothelial monolayer for 5 min at 37 °C, the presence of trypsin plus EDTA did not result in any significant increase in the radioactivity associated with the cells (for example, 0.15% in control, 0.14% with trypsin plus EDTA, means of three observations). Dipyridamole, a potent inhibitor of adenosine uptake by endothelial cells¹¹, was added to prevent uptake of any ^3H -adenosine formed during the incubation with ^3H -ATP,

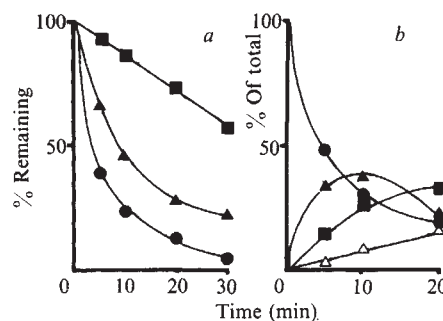


Fig. 2 Replicate samples of $\approx 10^5$ endothelial cells in culture were incubated with 1 μM ^3H -nucleotide. *a* Shows the fraction of substrate in serum-free HEPES-buffered Dulbecco's medium²³ remaining unchanged outside the cells at various times after incubation. *b* Shows the pattern of metabolites outside cells incubated with ATP in PBS containing 1.8 mM Ca^{2+} . ●, ATP; ▲, ADP; ■, AMP; △, adenosine. Dipyridamole (10 μM) was present in all experiments.

because we had confirmed (see below) the recent report¹⁵ that endothelial cells could metabolise extracellular ATP to adenosine.

It was clear that nucleotide metabolism occurred either during or after release, because the pattern of intracellular nucleotides secreted was (as shown in Fig. 1) different from that found outside the cells. This difference was even more striking when similar experiments were carried out using cultured pig aortic smooth muscle cells: up to 50% of the ³H secreted by smooth muscle cells was detectable after 5 min as ³H-adenosine, although again the main intracellular change was a loss of ³H-ATP. We did, however, establish that at shorter time intervals most of the label released was ATP. To investigate the extent of extracellular metabolism of adenine nucleotides in more detail, cultures of endothelial cells or smooth muscle cells were incubated with individual labelled nucleotides and the metabolites in the culture medium determined.

Figure 2a shows that endothelial cells catabolised added ATP, ADP or AMP and the half lives were, respectively, <5, ≈10 and >30 min at 1 μM substrate concentration. With smooth muscle cells the half life for each nucleotide was ≈5 min in the same conditions. Metabolism of added ³H-ATP by endothelial cells resulted in an initial transient peak of ³H-ADP, followed by a more prolonged peak of ³H-AMP and finally a progressive increase in ³H-adenosine (Fig. 2b). When the same experiment was carried out using smooth muscle cells, the peaks of ³H-ADP and ³H-AMP were much smaller and never exceeded the amount of adenosine formed.

Taken together, these results suggest sequential degradation of extracellular nucleotides (ATP→ADP→AMP→adenosine) by both endothelial cells and smooth muscle cells, although the relative rates of breakdown differ significantly in the two cell types. The pattern of extracellular nucleotides found after 5 min trypsinisation of each cell type is consistent with the transient release of ATP, and a smaller amount of ADP, followed by extracellular metabolism.

Our findings raise an important caveat for experiments with platelets and cultured cells. We, and others, have previously used a platelet aggregation bioassay to estimate the production of PGI₂ by cultured vascular cells, in which the cells are usually suspended by conventional procedures similar to those described in the present study—the use of trypsin, collagenase, EDTA or a rubber policeman^{1,2,8–10}. As we have now demonstrated a hitherto unsuspected selective release of adenine nucleotides from cultured vascular cells, affecting platelet aggregation, this must be considered in future experiments using this method.

The selective release of ATP from cultured endothelium seems to be a cellular response to potentially damaging stimuli. It is not a nonspecific increase in membrane permeability like that associated with cell lysis, because the cells remain viable and there is very little release of cytoplasmic protein. Nor is it apparently a secretory process involving degranulation—we found no evidence for compartmentalisation of endothelial ATP. We also failed to demonstrate any inward transport of ATP across the endothelial plasma membrane; this implies that the selective release may represent a unidirectional increase in membrane permeability to nucleotides. The release process could be in some respects analogous to the selective release of prostaglandins from stimulated cells, but the precise mechanism involved remains to be determined.

Whatever the mechanism responsible, the release of nucleotides from vascular endothelium *in vivo* would have great biological significance, because adenine nucleotides and adenosine not only influence platelet aggregation but are also potent vasoactive agents. Efflux of adenosine, with smaller quantities of nucleotides, has been measured in several perfused vascular preparations^{16–18}, particularly in the vasculature of cardiac or skeletal muscle during hypoxia, where the circulating adenosine is thought to act as a local hormone to increase blood flow^{19–21}. Although adenosine itself might be released from endothelium or muscle cells, perhaps by the uptake process

operating in reverse, this seems unlikely, as the intracellular concentration of adenosine is very low (<1% of the ATP concentration), and the reactive hyperaemia caused by endogenous adenosine during hypoxia is potentiated by inhibitors of adenosine transport such as dipyrindimole²². An alternative pathway would be the selective release of a small proportion of the cells' ATP with subsequent conversion to adenosine by the ectoenzymatic cascade. Further work is needed to test whether this pathway, which we have observed *in vitro*, operates *in vivo* (for example, in response to hypoxia) to regulate vascular tone.

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Orthotopic bone induction at sites of Moloney murine sarcoma virus inoculation in mice

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Moloney murine sarcoma virus (M-MSV) induces at the site of inoculation in newborn^{1,2} and adult^{3–5} mice and rats various types of sarcoma^{1,6}, including osteosarcoma^{7,8}. The induced tumours are fatal in newborn mice^{3,9}, whereas sarcomas developed in adult animals regress spontaneously^{3,5,10–12}. The regression is mediated mainly by a cellular response^{3,5,10–12}. We have now demonstrated that the presence of sarcomas induced by M-MSV is a powerful stimulus for periosteal osteogenesis around tumour masses. Orthotopically induced osteogenesis by M-MSV may serve as a model for local regulation of bone growth and for cell differentiation studies, and may help explain the aetiology of some human bone disorders.

Inbred 5-week-old NMRI mice of both sexes were inoculated into left shanks with 0.2 ml of standard M-MSV diluted 1:5 in phosphate-buffered saline. The virus was prepared according to Chanaille *et al.*¹³ (10³ TID₅₀ in 0.2 ml). Mice were killed at various intervals between the 3rd and 190th days after inoculation and both shanks, one inoculated with M-MSV and the intact contralateral control, were fixed in Susa solution. Paraffin

Fig. 1 Periosteal cancellous bone formation in ulna, 16 days after M-MSV inoculation. Arrows indicate the original width of the ulna cortex. $\times 60$.

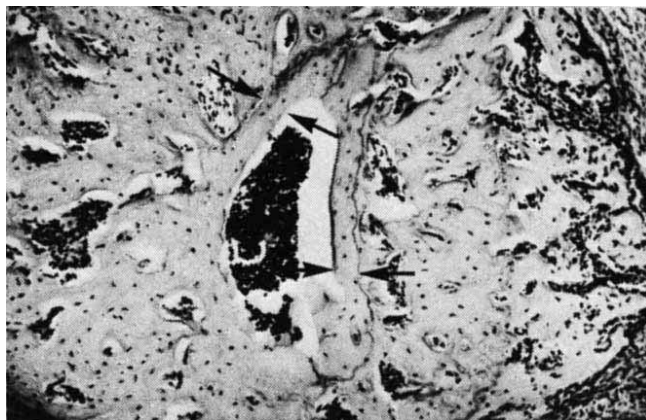


Fig. 2 Tibia, 2 months after M-MSV inoculation and 1 month after tumour regression. Arrows indicate the original width of the tibia cortex. $\times 38$.

sections from the middle of the shanks were cut perpendicularly to the tibial axis, and stained with haematoxylin and eosin. As an additional control five mice were inoculated with heat-inactivated M-MSV (heated at 56 °C for 2 h).

Of 36 mice inoculated with active M-MSV, 34 developed tumours (95%). Tumours grew progressively up to days 14–16, then declined and disappeared completely by days 25–32. Tumours were pleomorphic and had large elongated and round cells with a single hypertrophic nucleus and distinct nucleoli, small fibrosarcoma-like cells predominated. Neoplastic cells were infiltrated with numerous neutrophils. A massive destruction of muscles was always observed during the progressive phase of tumour growth. The regression of tumours was associated with regeneration of muscles, the disappearance of neoplastic cells, and the accumulation of macrophages, fibroblasts and lymphocytes. No heterotopic osteogenesis was observed either during progressive tumour growth or during regression.

A virus-induced sarcoma acts as a very powerful stimulus for orthotopic periosteal osteogenesis. In all cases the presence of tumours was associated with new bone formation in the periosteum of the tibia and/or ulna. In control bones the periosteum was composed of two to four layers of fibroblasts. At 6 days after M-MSV inoculation the periosteum became a thick membrane, composed of 7–20 layers of osteoblasts and fibroblasts; mitotic forms were frequent and osteoid deposition had begun. Bone formation continued until day 16. By this time the newly formed cancellous bone covering the old original cortex was three to five times thicker than the original bone (Fig. 1). The orthotopic bone formation was uneven, sometimes affecting the whole periphery, but always taking place on sites which faced the tumorous tissue. Newly formed orthotopic bone ossicles were also sites of myelopoiesis. Bone formation ceased with tumour regression. During tumour regression the remodelling processes converted induced trabecular bone into more mature forms.

Orthotopically induced bone also stopped growing and became covered with periosteal membrane of normal histology (Fig. 2).

Tumours did not form in shanks inoculated with heat-inactivated M-MSV, and there was no periosteal stimulation or bone formation. In the two mice in which inoculation of active M-MSV did not produce tumours, no changes in bone and periosteum histology were observed.

Orthotopically induced bone persisted throughout the 6-month observation period, remained unresorbed, and did not exhibit neoplastic changes. The results obtained may explain some aspects of the aetiology of osseous deformation, such as exostoses. It is not clear whether proliferation of the periosteum is caused by the virus itself, or whether it is mediated by tumour and/or inflammatory cells. M-MSV does not induce osteosarcomas when administered intramuscularly, but when administered into the medullary cavity of newborn rat tibia the virus induces highly malignant osteosarcomas⁸. The development of tumours precedes the stimulation of the periosteum, however, which suggests a direct mechanism of periosteal stimulation.

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Growth of a human mammary tumour cell line in a serum-free medium

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The MCF-7 line of human mammary carcinoma cells is a well characterised line¹ which has retained a number of the properties expressed by breast epithelium *in vivo*, making it a good model for the *in vitro* study of normal and neoplastic mammary cells. MCF-7 cells have been reported to possess receptors for oestrogen, androgen, progesterone, glucocorticoid², insulin³ and L-3, 3', 5-triiodothyronine (T₃)⁴. These cells also show responses to prolactin⁵, and dome formation¹. This line, like most cells in culture, requires the addition of a serum supplement to the medium for growth. The complex and undefined nature of serum adds a complicating aspect to the design and interpretation of any experiments aimed at understanding the interactions of hormones or drugs with mammary epithelium. Our laboratory has shown that it is possible to replace the serum requirement for a number of cell lines with mixtures of hormones and purified serum factors⁶⁻⁸. We now report that MCF-7 cells may be grown, without a lag or adaptation phase, in a serum-free medium supplemented with physiological levels of insulin, transferrin, epidermal growth factor (EGF), prostaglandin F_{2α} (PGF_{2α}) and cold-insoluble globulin (CIg). This medium will support a growth rate identical to that of cells in medium supplemented with an optimal concentration of fetal calf serum. The further addition of the α-1 serum protein first purified by Holmes⁹ results in a morphology identical to that of cells in 10% serum. Turkington has previously shown EGF effects on mouse mammary explant cultures¹⁰, and mouse EGF has been reported to be stimulatory for human mammary tumour cells¹¹. Insulin and transferrin have been shown to be required for the continuous serum-free growth of the ZR-75-1 human mammary tumour cell line¹². We believe that this is the first report of the effects of transferrin, PGF_{2α}, CIg, EGF or the α-1 protein on the MCF-7 line.

Figure 1 shows the growth over an 8-d period of MCF-7 cells in medium supplemented with 10% fetal calf serum or with the five factors. The doubling time in both conditions was about 36 h. This generation time agrees with that reported by other laboratories for these cells¹³. Addition of the five factors to cells in medium with 10% fetal calf serum did not increase cell number beyond that seen with 10% serum alone (data not shown). In serum-free medium in the absence of the five factors

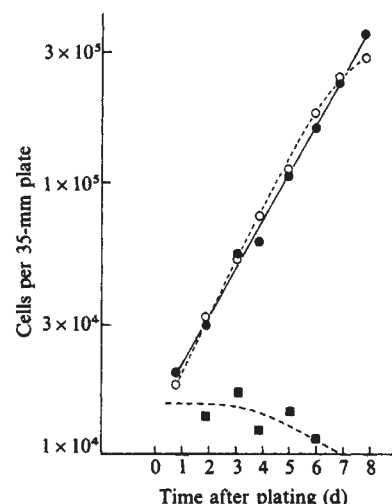


Fig. 1 Growth of MCF-7 cells in serum-free medium. Stock cultures for experiments were grown in a one to one mixture of F12 and DMEM supplemented with antibiotics, 15 mM HEPES pH 7.4, 1.2 g l⁻¹ sodium bicarbonate and 10% fetal calf serum (Reheis). Cells were detached from stock flasks with a trypsin-EDTA solution in phosphate-buffered saline, treated with trypsin inhibitor, and plated at 3×10^4 cells per 35-mm diameter plate in 2 ml medium supplemented with 250 ng ml⁻¹ insulin (Sigma), 25 µg ml⁻¹ transferrin (Sigma), 100 ng ml⁻¹ EGF (Collaborative Research), 100 ng ml⁻¹ PGF_{2α} (Sigma) and 7.5 µg ml⁻¹ CIg (Collaborative Research) (O). Control plates contained medium with 10% fetal calf serum (●), or medium with no stimulatory supplements (■). Data shown are the average of triplicate samples. Deviation of single samples from the average was about 5%. For counting, cells were treated with trypsin-EDTA, sheared through a 22-gauge hypodermic needle to produce a single cell suspension, and counted in a Coulter counter. Cell suspensions treated in this way gave counts identical to those counted with a haemocytometer.

cell death occurred after 4 d. Cell growth in the serum-free medium began to slow 6 d after plating and fell behind that of serum by day 8. In most experiments cell number in serum-supplemented medium at any point in the growth curve was higher than that of cells in the serum-free medium. This reflected a higher initial plating efficiency in serum-supplemented medium compared with serum-free medium, but did not represent a significantly lower doubling time in the serum-free medium. The synthetic nutrient medium used in these experiments was a 1:1 mixture of Ham's F12 and Dulbecco-modified Eagle's medium (DMEM) supplemented with antibiotics, 1.2 g l⁻¹ sodium bicarbonate, 15 mM HEPES, pH 7.4, and 10⁻⁸ M sodium selenite. No growth was seen in DMEM supplemented with the five factors, and growth in F12 supplemented with the five factors was 80% of that seen in the mixture of the two.

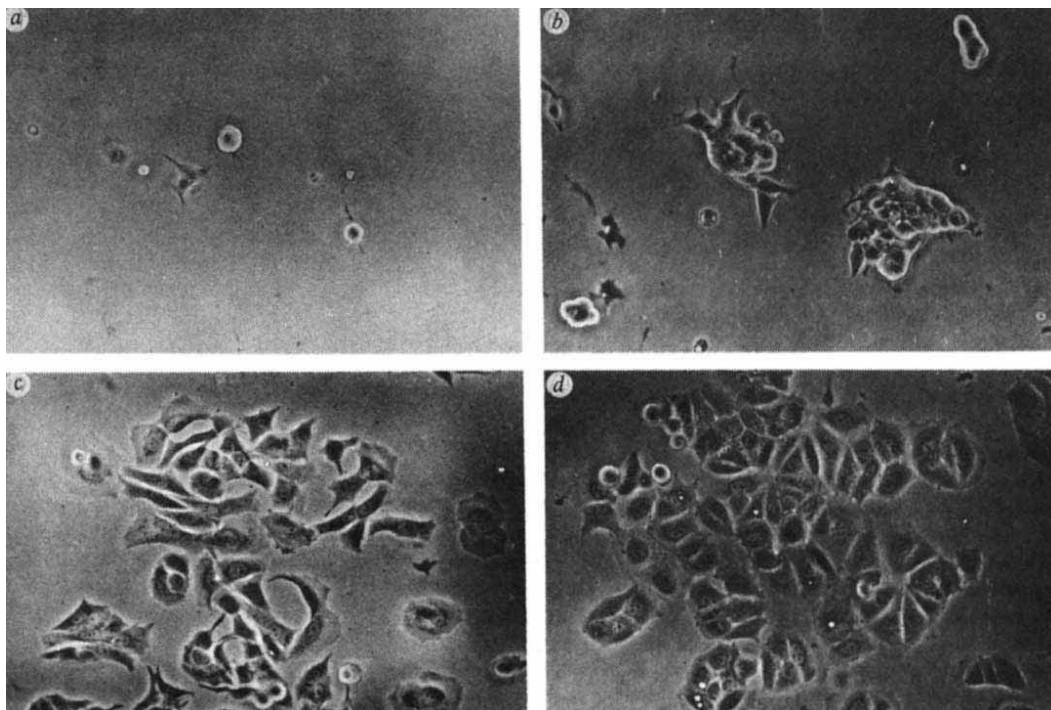
The individual omission of either insulin, transferrin, PGF_{2α}, or CIg from the medium resulted in decreased cell number after 7 d (Table 1). Longer incubations (10–14 d) were required to demonstrate EGF effects. EGF effects could not be observed at earlier times if insulin was omitted or the insulin concentration lowered. Insulin was clearly the most important component of the defined medium. In dose-response studies carried out in the presence of the other factors, the maximal effect of insulin was observed with 100–300 ng ml⁻¹ with an easily measurable effect at 3 ng ml⁻¹. Transferrin produced a maximal stimulation at 25 µg ml⁻¹ with a measurable effect at 1 µg ml⁻¹. The CIg effect was maximal at 7.5 µg ml⁻¹ with a measurable effect at 0.75 µg ml⁻¹. CIg at these concentrations is also stimulatory for the RF-1 rat ovary cell line¹⁴ and a mouse embryonal carcinoma line¹⁵. The effect of CIg on cell number in Table 1 was not due to decreased shedding of cells into the medium. The number of cells floating in the medium was less than 2% of the total number in the dish and this percentage was unchanged by omission of CIg from the medium. The transferrin concentration used in the

Table 1 Effect of insulin, transferrin, PGF_{2α}, EGF and CIg on growth of MCF-7 cells in serum-free medium

Conditions	Cells per 35-mm plate ($\times 10^{-4}$)
No additions	4.4 ± 0.1
Insulin, transferrin, EGF, PGF _{2α} , CIg	35.0 ± 0.9
Insulin omitted	11.4 ± 1.4
Transferrin omitted	28.4 ± 2.3
PGF _{2α} omitted	30.4 ± 2.0
CIg omitted	27.7 ± 1.0
EGF omitted	34.6 ± 2.4
10% fetal calf serum	40.0 ± 2.8

Cells were plated at 3×10^4 per 35-mm diameter plate in medium with the indicated additions. Concentrations of the factors were the same as that of Fig. 1. Cells were counted 7 d after plating. After 14 d (medium changed on day 8), cell number in medium with the five factors was 85.1×10^4 per plate. In medium with EGF omitted cell number was 78.8×10^4 per plate.

Fig. 2 Effect of the α -1 protein of Holmes on morphology of MCF-7 cells in serum-free medium. Cells were incubated for 3 d in (a) medium with no stimulatory supplements, (b) medium with the five factors as described in Fig. 1, (c) medium with five factors plus the α -1 protein (GIBCO), and (d) medium with 10% fetal calf serum. The human α -1 protein was used at a dilution of 1:10. Protein concentration of the α -1 protein in the medium, calculated from information provided by the vendor, was about 250 ng ml^{-1} .



serum-free medium ($25 \mu\text{g ml}^{-1}$) is about 1/10th (ref. 16), and the CIG concentration ($7.5 \mu\text{g/ml}^{-1}$) about one-third¹⁷, that which would be found in medium containing 10% serum. $\text{PGF}_{2\alpha}$ was maximally stimulatory at 100 ng ml^{-1} and EGF at 10 – 100 ng ml^{-1} . Both of these factors showed some stimulation at 1 ng ml^{-1} . No increase in cell number was seen on addition of physiological levels of other hormones which have effects on mammary epithelia, including human prolactin, porcine relaxin and vasopressin.

Cells in serum-free medium with the five factors grew as rounded clumps which were poorly attached to the dish, or groups of cells which were attached but poorly spread. The addition of the α -1 serum protein, which has been reported to enhance the growth and spreading of HeLa cells⁹, resulted in a striking change in morphology (Fig. 2). This protein greatly improved attachment and spreading of MCF-7 in serum-free medium at concentrations of $1 \mu\text{g ml}^{-1}$ or less.

Increased cell number has been reported for MCF-7 under various circumstances upon addition of insulin¹⁸, oestrogen¹⁹, androgen²⁰ or T_3 (ref. 4). The androgen effect is reported to be mediated by the oestrogen receptor²¹. Morphological effects of both T_3 and oestradiol at concentrations as low as 10^{-10} M were observed in our medium containing the five factors, but no increase in cell number was seen after incubations as long as 14 d, with a medium change on day 7. However, additions of oestradiol at 10^{-9} M did result in an increase in cell number if the oestrogen was added to cells plated in the α -1 serum protein alone (W. Desmond, D. B. and G. S. in preparation). Although it is possible that long-term cultivation of MCF-7 cells in our serum-free medium may expose additional requirements for other hormones, these cells have so far been successfully carried for over 3 months (15 passages) in the serum-free medium.

If great care is taken on transfer to insure removal of residual trypsin, proper control of medium pH and temperature and to minimise the amount of time they are out of the incubator, the cells may be continually passaged in the serum-free medium without loss of viability or lengthening of generation time. When cells were grown for five passages in the serum-free medium and then plated in medium with 10% fetal calf serum or medium with the five factors, cell number after 6 d in plates with medium with the five factors was 80% of the cell number in plates with 10% serum.

Allegra and Lippman¹² have described a serum-free medium for ZR-75-1 human mammary tumour cells which includes insulin, transferrin, dexamethasone, T_3 and oestradiol. This

medium will not support the growth of MCF-7. Although it is not known if the ZR-75-1 line will grow in the medium devised for MCF-7, preliminary experiments indicate that, in addition to MCF-7, another human mammary carcinoma cell line, BT20, also grew in serum-free medium supplemented with insulin, transferrin, EGF, $\text{PGF}_{2\alpha}$, CIG, and the α -1 protein.

A serum-free medium for MCF-7 cells should be useful for several reasons. This medium will greatly facilitate pharmacological and nutritional studies of MCF-7. Studies of hormone binding and subsequent biological effects may now be carried out on healthy, growing, non-selected cells in the absence of interfering hormones or serum proteins. Serum-free medium will allow the easy identification and isolation of products synthesised and secreted by the cells into the medium. Finally, a serum-free medium for human mammary tumour cells may allow the isolation of new lines of normal or neoplastic human mammary epithelium in the absence of other contaminating cell types.

We thank Dr C. McGrath and Dr W. McGuire for gifts of MCF-7 cells. Experiments described were carried out with MCF-7 cells supplied by Dr McGuire. MCF-7 cells provided by Dr McGrath also grew in the serum-free medium. We also thank Dr D. McClure, Dr W. Desmond and Dr G. Rockwell for critical reading of the manuscript and many helpful discussions of the data. This work was supported by a NCI postdoctoral grant IF32CA06188-01 and a NCI contract N01-CB74188.

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Platelet-derived growth factor prevents G_0 growth arrest

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Baserga¹ has summarised evidence that there are two growth states in which cells have a diploid content of DNA; G_1 —the interval between mitosis and S phase in exponentially growing cultures—and G_0 , a quiescent state entered only when conditions for growth are suboptimal. G_0 can be distinguished from G_1 by temporal measurements; for BALB/c 3T3 cells, the lag time between G_0 and S phase is never shorter than 12 h (ref. 2), whereas in exponentially growing cells the mean lag time between mitosis and S (G_1 by definition) lasts 5–6 h (ref. 3). G_0 can also be distinguished from G_1 by some biochemical parameters including G_0 - and G_1 -specific intracellular proteins^{4,5}. SV40- or polyoma virus-transformed cells cannot enter G_0 to become quiescent^{6–9}. Serum induces the growth of BALB/c 3T3 cells in tissue culture. It sustains the growth of exponentially replicating populations, and causes density-inhibited cells to leave G_0 and replicate^{10–12}. Serum contains several sets of hormonal growth factors which have recently been defined^{2,13,14}. One of these hormones, the platelet-derived growth factor (PDGF), is released into serum during the clotting process; PDGF is absent in platelet-poor plasma, the liquid portion of unclotted blood^{15–19}. It promotes the growth of G_0 -arrested cells^{2,16} by stimulating cells to become 'competent' to enter the S phase²; plasma allows these competent cells to progress through G_0/G_1 , synthesise DNA^{2,20} and divide¹³. We now show that PDGF has a second function. It prevents replicating cells from entering G_0 .

Density-inhibited cultures of BALB/c 3T3 cells (clone A31) were treated for 5 h with platelet extract containing partially purified PDGF to induce the majority of the cells to become competent for replication. Transfer of these cells to medium containing an optimal concentration (5%) of dialysed platelet-poor plasma allows these cells to begin to enter the S phase after a lag of 12 h (refs 2, 20, 21). Addition of the dihydrofolate reductase inhibitor methotrexate²² (100 nM) prevented DNA synthesis, and flow microfluorimetry demonstrated that the growth-arrested cells had an apparent G_1 content of DNA (data not shown). The methotrexate was removed and plasma-supplemented medium containing hypoxanthine and thymidine was added; duplicate cultures were treated with ³H-thymidine (TdR) or colchicine for 1 h, at various times after removal of the methotrexate, and fixed². DNA synthesis, as determined by autoradiography, was detected in 87% of the cells within the first hour after removal of the methotrexate (Fig. 1). The percentage of cells synthesising DNA remained constant for 6 h and then fell abruptly. By 10 h, little DNA synthesis was detected, indicating that the S phase had been completed. Mitosis was somewhat less synchronous, beginning at 9 h, reaching a peak at 11 and ending by 15 h. Treatment of cells with PDGF after the removal of methotrexate did not alter the kinetics of DNA synthesis or mitosis (data not shown).

Cells arrested by plasma withdrawal shortly before the onset of DNA synthesis enter the S phase with first-order kinetics, whereas cells blocked in early S phase begin DNA synthesis in a synchronous fashion^{20,23}. Thus, the data indicate that methotrexate blocks cells near the G_1 /S phase boundary in early S phase.

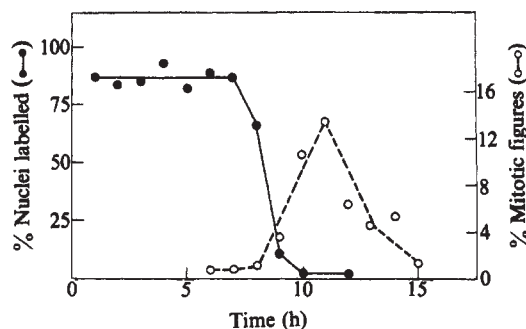


Fig. 1 Methotrexate causes growth arrest in early S phase. BALB/c 3T3 cells (clone A31) were grown to confluence on Falcon microtitre wells (area 0.3 cm²) in Dulbecco's modified Eagle's medium containing 10% calf serum². Density-inhibited cultures were treated with 150 µg ml⁻¹ of heat (100 °C)-treated platelet extract containing PDGF for 5 h, washed once and transferred to medium containing 5% (v/v) dialysed human plasma containing 100 nM methotrexate (National Cancer Institute). After 18 h all cultures were transferred to fresh medium containing 5% plasma, 106 nM hypoxanthine and 16 nM thymidine. ³H-TdR (5 µCi ml⁻¹) was added to duplicate cultures at hourly intervals after removal of the methotrexate. The cells were fixed 1 h after addition of ³H-TdR at the time shown, washed twice with 5% trichloroacetic acid and processed for autoradiography. Duplicate cultures were treated with 2 µM colchicine (GIBCO) for 1 h and fixed at the time shown; the percentage of mitotic figures was determined.

Density-inhibited BALB/c 3T3 cells were treated first with platelet extract containing PDGF and then with 5% plasma containing methotrexate as above; 18 h later, when 90% of the cells were growth arrested in early S phase, the methotrexate block was removed. ³H-TdR was added to the cultures 16 h after removal of the methotrexate to study DNA synthesis in the post-mitotic daughter cells. Without further PDGF treatment, <10% of the daughter cells entered the S phase within the 27 h period after removal of the methotrexate block (Table 1). Addition of partially purified PDGF to the parent cells for 2-h periods during the first 8 h after removal of the methotrexate (corresponding largely to parental cell S and G_2) induced 60–70% of the daughter cells to synthesise DNA (Table 1). The

Table 1 Treatment with PDGF during the S phase stimulates post-mitotic daughter cells to synthesise DNA

Interval (h) treated with PDGF after methotrexate removal	% Of daughter cells synthesising DNA
0–2	67
2–4	68
4–6	70
6–8	59
Untreated	9

Density-inhibited BALB/c 3T3 cells were treated with 150 µg ml⁻¹ of heat-treated platelet extract containing PDGF for 5 h, washed once and transferred to medium containing 5% (v/v) dialysed human plasma containing 106 nM methotrexate. After 18 h all cultures were transferred to fresh medium containing 5% plasma, 100 nM hypoxanthine and 16 nM thymidine. Addition of ³H-TdR (5 µCi ml⁻¹, final concentration 0.3 Ci mmol⁻¹) from 0–2 h after the methotrexate was removed, followed by immediate fixation and autoradiography, demonstrated that 90% of the parent cells synthesised DNA. Duplicate cultures were treated with heat-treated platelet extract (150 µg ml⁻¹) in the presence of hypoxanthine and thymidine, but in the absence of plasma for the interval shown (or were left untreated), washed twice with medium and returned to the plasma-supplemented medium containing hypoxanthine and thymidine. Approximately 16 h after the methotrexate was removed, ³H-TdR was added; the cultures were fixed and processed for autoradiography 11 h later and the percentage of post-mitotic daughter cells entering the S phase was determined.

percentage of PDGF-treated daughter cells that entered the S phase was also controlled by the concentration of plasma in the medium; in 0.25% plasma 30% of the daughter cells entered the S phase, compared with 90% in 5% plasma.

To study the effect of the PDGF concentration on the percentage of post-mitotic daughter cells stimulated to enter the S phase, platelet extract was briefly added to parental cells undergoing DNA synthesis. The percentage of daughter cells stimulated to synthesise DNA increased approximately linearly as a function of the logarithm of the platelet extract concentration over a 10-fold range (Fig. 2a). Treatment of parent cells with pure PDGF¹⁹ also stimulated the daughter cells to synthesise DNA in a dose-dependent fashion (Fig. 2b). A 2-h treatment with 5 ng ml⁻¹ of a homogeneous preparation of PDGF stimulated 50% of the daughter cells to enter the S phase.

To study the lag phase until DNA synthesis in post-mitotic daughter cells, duplicate cultures were released from the methotrexate block and briefly treated with partially purified PDGF. Treatment of the parent cells with PDGF from 0–2 or 4–6 h after release of the methotrexate block (corresponding to early or middle S phase) induced the daughter cells to begin synthesising DNA 15 h from the release of the methotrexate block (Fig. 3a, b), approximately 6 h after the beginning of mitosis. Addition of PDGF from 8–10 h after methotrexate removal (corresponding largely to parental G₂ and M phases) stimulated the daughter cells to enter the S phase at 17 h, approximately 7 h after the PDGF treatment was completed and the cells were returned to plasma (Fig. 3c). Addition of PDGF to the post-mitotic daughter cells, 12–14 h after the methotrexate was removed largely corresponding to G₁, 3–5 h after the beginning of mitosis, induced the cells to enter the S phase 7 h after the PDGF treatment (Fig. 3d). However, addition of the PDGF to the post-mitotic daughter cells 16–18 h after methotrexate was removed (7–9 h after the beginning of mitosis) resulted in a significant delay in S phase entry (Fig. 3e). These cells began to enter the S phase 12 h after PDGF was removed, demonstrating that they had become arrested in the G₀ phase of the cell cycle.

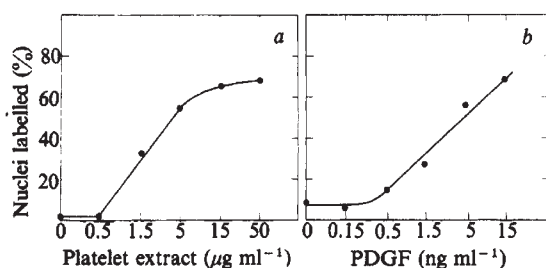


Fig. 2 The concentration of PDGF regulates the percentage of post-mitotic daughter cells synthesising DNA. Density-inhibited BALB/c 3T3 cells were treated with partially purified PDGF and transferred to 5% plasma-supplemented medium containing methotrexate as outlined in Fig. 1 legend. After 18 h the medium was removed from all cultures; some cultures were transferred to medium containing 5% plasma, hypoxanthine and ³H-TdR (5 μCi ml⁻¹; 0.3 Ci mmol⁻¹) and fixed 8 h later. Autoradiography demonstrated that 90% of the PDGF-stimulated cells and <1% of the unstimulated cells entered the S phase. Duplicate cultures were treated with heat-treated platelet extract (a), or pure PDGF which was purified to homogeneity from clinically outdated human platelets as has been described elsewhere¹⁹ (b), at the concentrations shown, plus thymidine and hypoxanthine. Two hours later the PDGF source was removed, the cultures were washed and transferred to 5% plasma-supplemented medium containing hypoxanthine and thymidine. ³H-TdR was added to the cultures 10 h after removal of the platelet extracts. The cultures were fixed and processed for autoradiography 16 h later. The lag phase for DNA synthesis is 12 h after plasma addition for the G₀-arrested cultures², and 13 h for the second wave of DNA synthesis (Fig. 3a) in the S phase cultures.

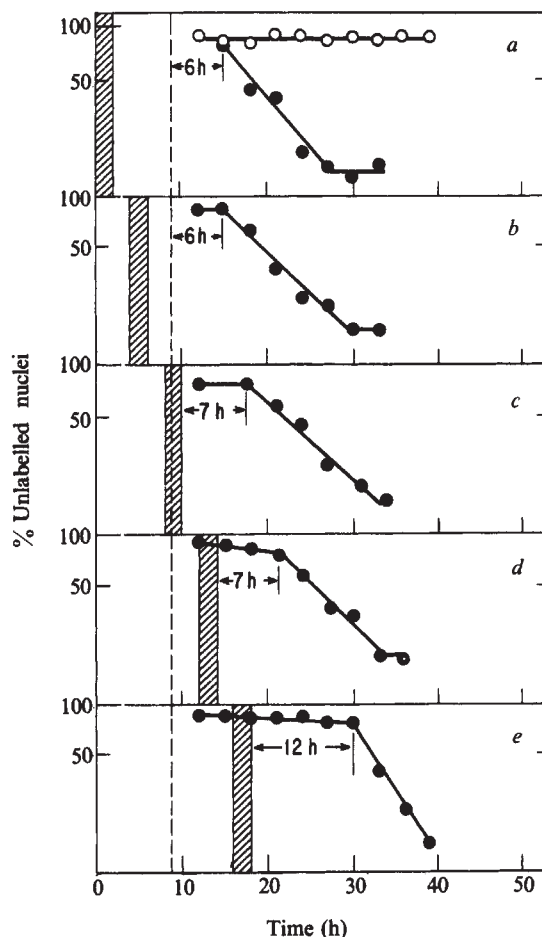


Fig. 3 Treatment with PDGF during S, G₂ or M phases, or immediately after mitosis, prevents replicating BALB/c 3T3 cells from becoming growth arrested at G₀. Density inhibited BALB/c 3T3 cells were treated with partially purified PDGF and transferred to 5% plasma-supplemented medium containing methotrexate as outlined in Fig. 1 legend. After 18 h, the medium was removed from all cultures; some cultures were transferred to medium containing 5% plasma, hypoxanthine and ³H-TdR (5 μCi ml⁻¹; 0.3 Ci mmol⁻¹) and fixed 8 h later. Autoradiography demonstrated that 91% of the PDGF-stimulated parent cells entered the S phase. Duplicate cultures were transferred to 5% platelet-poor plasma containing hypoxanthine and thymidine. Some cultures (●) were treated with platelet extract (150 μg ml⁻¹) containing partially purified PDGF and hypoxanthine and thymidine for 2-h periods at the times indicated by the shaded bars or were left untreated (○). ³H-TdR (5 μCi ml⁻¹; final concentration 0.3 Ci mmol⁻¹) was added to these cultures 10 h after the removal of the methotrexate. The cells were fixed at the indicated times and processed for autoradiography. The data are plotted according to the method of Smith and Martin²⁴. The dashed line is shown to mark the beginning of mitosis (see Fig. 1).

The present data clarify the role of PDGF in regulating fibroblast growth by demonstrating that this hormone has two activities. PDGF can act during the late stages of the cell cycle, or shortly after mitosis, to prevent BALB/c 3T3 cells from entering G₀. It can also act on G₀-arrested cells to promote exit from the quiescent state. A brief treatment of quiescent cells with PDGF stimulates a single replicative cycle.

Treatment of parental cells with PDGF, during or after the S phase, allows the daughter cells to synthesise DNA. One possibility is that PDGF stimulates 'G₀-specific' events concomitantly with S, G₂ and M phases. In such a case, G₀ might not represent a true growth arrest state, but would merely reflect a series of time-dependent events which must occur in all cell cycles before DNA synthesis. The increased lag period noted in post-mitotic quiescent cells would represent the time required to complete

these events. However, the addition of PDGF to the daughter cells immediately after parental mitosis also prevents growth arrest, with cells entering the S phase after a short lag period (Fig. 3d). Further delay in the addition of PDGF causes cells to become quiescent (Fig. 3e). Thus, cells enter G_0 if they are not treated with PDGF before a critical period in the replicative cycle, approximately 3–5 h after the beginning of mitosis. Addition of PDGF after this period does not prevent entry into G_0 ,

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but releases cells from G_0 , a process which requires an additional 5 h before entry into the S phase. Clearly, PDGF does not simply cause ' G_0 -specific' events to be telescoped into the preceding cell cycle. The evidence supports the concept that G_0 is a distinct growth arrest state which cells can enter some time after mitosis.

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Metabolites of diethylstilboestrol induce sister chromatid exchange in human cultured fibroblasts

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Diethylstilboestrol (DES) is one of the few substances for which a clear association with carcinogenicity has been established in man^{1,2}. Nevertheless, it is still widely used, mainly as a cheap oestrogen to increase the slaughter weight of beef³, but in spite of this it is not known if residues in the meat or metabolites excreted by the cattle are hazardous to man. It is also unknown whether there is a threshold dose below which DES is harmless. A threshold might be expected if a hormonal mechanism of carcinogenesis rather than metabolic activation to an electrophilically reactive species operates. This possibility was supported by the observations that DES, in contrast to most other carcinogens, failed to induce mutations in the *Salmonella*/microsome test^{4–6} or malignant transformations of eukaryotic cells in culture⁷. It is also disturbing that DES, one of the few known human carcinogens was negative in these two most widely used short-term tests introduced as fast early-warning systems for potential carcinogens. We now report that DES is positive in sister chromatid exchange (SCE) induction, a short-term test for which a high correlation with the carcinogenicity of the compounds tested has been observed^{8–10}. Moreover, we show that metabolic activation was involved. Two different pathways leading to metabolites much more active in SCE induction than DES itself ('proximate agents') were established.

DES- α , β -oxide and β -dienoestrol (Fig. 1) were synthesised as described previously¹¹. The mutagenicity test was performed as described by Ames¹². As mammalian metabolising system S-9 mix¹² from liver homogenate of Aroclor 1254-treated male Sprague-Dawley rats (200–300 g) was used. Fibroblast cultures were established¹³ from skin biopsies of the inner side of the

upper arm of healthy male and female volunteers of Caucasian origin. 5-Bromodeoxyuridine ($30 \mu\text{g ml}^{-1}$) and the test compound were incubated with the cells for 72 h. After staining with acridine orange¹⁴, SCE was directly evaluated under a fluorescent microscope.

The initial experiment revealed that DES induces SCE. The number of SCEs was increased even at low doses with a further shallow increase as the DES concentration is raised (Fig. 2, open symbols). The results obtained with diploid fibroblast cultures of five different donors were very similar. Interestingly, lower concentrations of DES were sufficient to cause SCE than of the positive control benzo (α) pyrene (Table 1). To clarify whether DES induces SCE itself or whether it requires metabolic activation, we used two methods—inhibition of metabolism and test of metabolites for induction of SCE. Several pathways of DES metabolism are known^{11,15}. All include some chemically reactive intermediates: (1) hydroxylation *ortho* to the phenolic hydroxyl groups leads to highly reactive catechols; (2) oxidation of the olefinic α , β -double bond produces an electrophilically reactive epoxide as the primary metabolite; (3) β -dienoestrol, a quantitatively important metabolite is formed by peroxidases, probably via highly reactive semiquinones and quinones. β -Dienoestrol can be further oxidised to ω -hydroxy- β -dienoestrol which can form reactive esters. At first, we tested two key metabolites, DES- α , β -oxide and β -dienoestrol (structures shown in Fig. 1). We observed an increase of SCE at even lower concentrations of test compound than with DES (Fig. 2). Dual reciprocal plots of the SCE induced against the concentration of

Table 1 SCE-induction by DES and metabolic intermediates

Test compound	Maximum SCE per metaphase	Concentration for half maximal induction for SCE*	Potency†
DES	11.0	133 nM	1
DES- α , β -oxide	18.2	40 nM	9.6
β -Dienoestrol	16.8	5 nM	70
Benzo(α)pyrene	15.7	2,100 nM	0.14

SCEs were investigated as described in Fig. 2 legend. Controls showed 7.2 ± 0.9 (s.d.) SCE per metaphase.

* Values are calculated from dual reciprocal plots by linear regression analysis. The r -values were >0.97 .

† The maximal number of SCE which were caused by the test compound (minus the number of spontaneous SCE) was divided by the concentration of test compound which caused the half-maximal effect. This value was divided by that of DES and is used as measure of the ability of the compound to cause SCE.

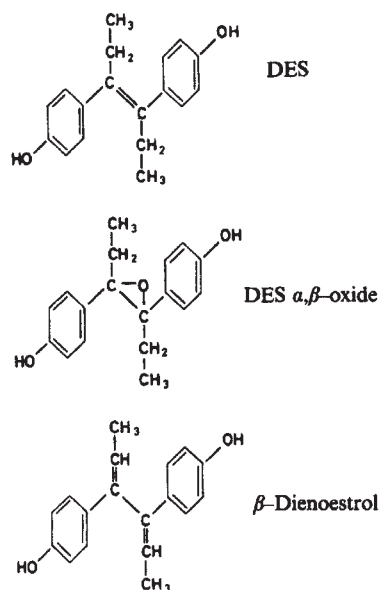


Fig. 1 Chemical structure of diethylstilboestrol (DES), and the two metabolites used for SCE induction.

inducing substance were used to calculate the maximum frequency of SCE and the concentrations for half maximal SCE induction (Table 1). The much higher potency of the metabolites strongly suggests that the induction of SCE by DES requires a metabolic activation. As we had previously shown¹⁶ that the metabolic activation of benzo(a)pyrene to a derivative inducing SCE can be inhibited by α -naphthoflavone, which inhibits mono-oxygenases¹⁷, we determined the SCE induction by DES and two metabolites in the presence of α -naphthoflavone (Fig. 3). The induction of SCE normally observed with DES was completely inhibited by α -naphthoflavone and was markedly reduced when α -naphthoflavone was added together with β -dienoestrol. The SCE-induction obtained with DES- α,β -oxide, however, could only be lowered about 35% by the presence of α -naphthoflavone. This provides additional evidence that DES and also β -dienoestrol require metabolic activation by mono-oxygenase for the induction of SCE.

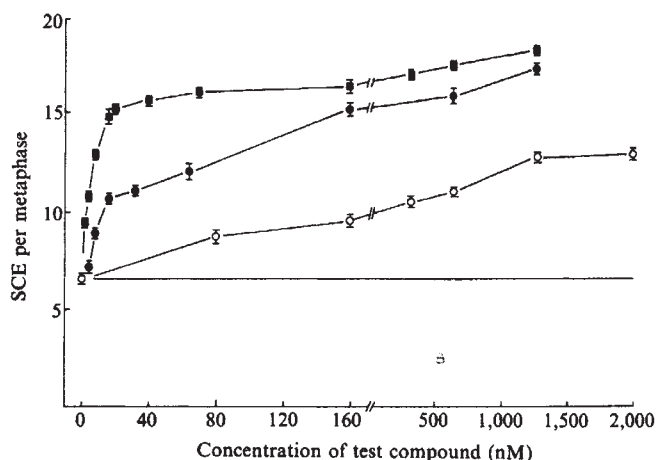


Fig. 2 SCE induction by DES (○) DES- α,β -oxide (●) and β -dienoestrol (■). The test compounds were dissolved in ethanol and then mixed with a twofold excess of sterile fetal bovine serum (FBS). Various amounts of these stock solutions (which were at least 100-fold concentrated as compared to the final dilution in the culture medium) were added to 25 cm² cell culture flasks (Falcon) containing 5×10^5 cells together with 5 ml Dulbecco's modified Eagles medium (GIBCO), supplemented with 10% FBS (GIBCO), 2×10^5 IU l⁻¹ penicillin, 0.1 g l⁻¹ neomycin and 0.1 g l⁻¹ streptomycin, and buffered with 20 mM HEPES and 0.5 g l⁻¹ NaHCO₃. The concentration of ethanol in the culture medium did not exceed 0.5%. Controls received 0.5% ethanol only.

Recently it was reported that DES can induce unscheduled DNA synthesis¹⁸ in HeLa cells, and induces mutations¹⁹ in a mouse lymphoma cell line. However, both effects could only be demonstrated over a very narrow, already markedly cytotoxic dose range and quantitative reproducibility was poor. Thus, neither test is suitable for studying factors which determine susceptibility towards DES. In contrast, SCE induction by DES in human diploid fibroblasts shown in this study did occur at very low doses and was dose-dependent over a wide range of concentrations. Thus the system could be used to investigate whether metabolic activation was involved, and to discover pathways leading to metabolites more active in SCE induction than DES itself ('proximate agents').

Table 2 Reversion of *his*⁻ *S. typhimurium* to histidine prototrophy

Test compound (μ g per plate)	S-9 mix	<i>his</i> ⁺ colonies per plate (range)			
		TA 100	TA 1535	TA 1537	TA 98
Solvent control	+	100-134	9-20	17-31	31-39
3.15-3000 DES	+	95-170	6-22	13-33	25-54
3.15-3000 DES α,β -oxide	+	89-141	7-24	5-24	23-41
3.15-315 β -Dienoestrol	+	85-149	13-19	10-27	34-63
10 Benzo(a)pyrene	+	980-1720	31-44	152-274	589-1,130
10 2-Aminoanthracene	+	5,500-9,100	568-668	240-318	6,200-6,700
90 3-Methylcholanthrene	+	5,000-8,900	12-23	50-97	1,180-1,520
Solvent control	-	153-192	17-30	8-13	11-21
3.15-315 DES	-	126-165	12-20	1-11	14-22
3.15-3000 DES α,β -oxide	-	144-200	8-22	5-15	15-24
3.15-315 β -Dienoestrol	-	139-189	13-23	4-7	11-21
1 Benzo(a)pyrene 4, 5-oxide	-	2,340-4,000	21-31	620-700	5,000-5,700
10 N-Methyl-N'-nitro-N-nitrosoguanidine	-	50,000-61,000	45,000-80,000	68-115	38-41

Histidine-dependent bacteria (*S. typhimurium* strain TA 100, TA 1535, TA 1537 or TA 98), test compound (dissolved in 10-30 μ l dimethyl sulphoxide with or without a mammalian metabolising system (S-9 mix from liver homogenate of Aroclor 1254-treated rats) were poured with a histidine-poor top agar on minimal agar plates. Colonies (*his*⁺ revertants) were counted after incubation for 3 d at 37 °C. DES and its metabolites were tested in duplicates at the following doses: 3.15; 10; 31.5; 100; 315; 1,000 and 3,000 μ g per plate. The table shows the range of the number of colonies at those doses where neither a significant reduction of the number of *his*⁺ colonies nor of the *his*⁻ background lawn was observed. Each individual experiment with S-9 mix contained benzo(a)pyrene, 2-aminoanthracene, 3-methylcholanthrene and solvent controls. Each experiment for direct mutagenicity contained benzo(a)pyrene 4, 5-oxide, N-Methyl-N'-nitro-N-nitrosoguanidine and solvent controls. Values for these controls represent ranges of all the experiments used for this study.

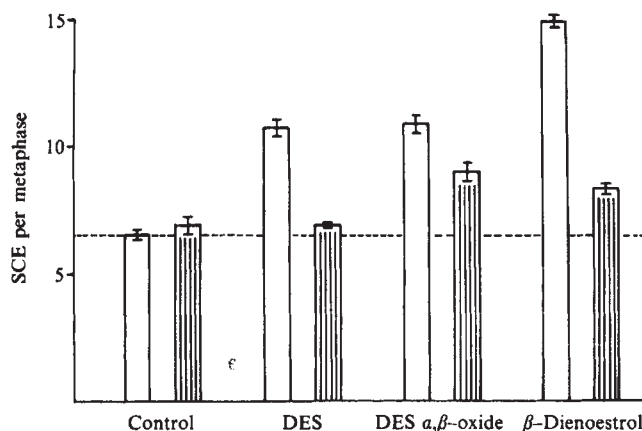


Fig. 3 Effect of α -naphthoflavone on the induction of SCE by DES, DES- α , β -oxide and β -dienoestrol. The experiments were performed as described in Fig. 2. α -Naphthoflavone was dissolved in acetone, mixed with a 40-fold excess of FBS and added to the culture. DES was present at 1.25 μ M, DES- α , β -oxide and β -dienoestrol at 20 nM. The bars show mean numbers of SCE per metaphase $\pm$ s.d. of incubations without (□) and with (▨) α -naphthoflavone (5 μ g ml $^{-1}$).

Note that the concentrations of test compound at which SCE were induced were low not only in absolute terms but also in relation to the toxicity of the compounds. With DES- α , β -oxide and β -dienoestrol cytotoxic effects that lead to an altered microscopic appearance of the cells and to a reduced number of metaphases were observed only at concentrations more than 100-fold greater than those needed for a marked SCE induction. The induction of SCE at doses which are much lower than those needed for observing toxicity favour the assumption that the exchanges of DNA fragments observed as SCE reflect specific DNA-directed damage rather than DNA alterations as an effect of broad disturbances of cell growth.

These results showed that some metabolites of DES are much more potent inducers of SCE than DES itself, so we investigated if these metabolites could induce mutations in *Salmonella typhimurium* either directly or after activation by mammalian tissue preparations. Table 2 shows that neither DES nor the two metabolites DES- α , β -oxide and β -dienoestrol induced any mutations to histidine prototrophy with *his* *S. typhimurium* strains in conditions where *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and benzo(*a*)pyrene-4,5-oxide (in the experiments for direct mutagenicity) and benzo(*a*)pyrene, 3-methylcholanthrene and 2-aminoanthracene (in the presence of S-9 mix) strongly increased the number of *his*⁺ revertant colonies.

Our results show that from DES at least two metabolic routes lead to metabolites which cause SCE: (1) via or to DES- α , β -oxide; (2) via β -dienoestrol which is further activated by monooxygenase. The activation via β -dienoestrol led to much stronger SCE effects than the activation via DES- α , β -oxide. In this context it is interesting that organs susceptible to DES carcinogenesis possess high activities of peroxidase²⁰, which metabolises DES to β -dienoestrol. The demonstration of metabolic activation of DES for SCE induction does, of course, not exclude that hormonal mechanisms and stimulation of cell proliferation may be important contributors to its carcinogenic effects.

The reasons why results with SCE are not reproduced in the Ames test are not clear. It may be that the *S. typhimurium* assay cannot detect mutations caused by DES metabolites as it is a back-mutation assay or because the exchange of identical genetic material during the SCE may not lead to mutation. Alternatively, metabolic factors may be involved—for example, enzymes (such as conjugating enzymes) may be required for the activation of DES which are active in human fibroblasts but not in the dilute postmitochondrial liver fraction in the plates of the

bacterial mutagenicity test. Or conversely, enzymes may be present in the S-9 mix which efficiently inactivate the active DES-metabolites.

The induction of SCE in fibroblasts by DES is an *in vitro* system which will be of value as a tool for the study of the mechanisms of activation and inactivation of DES and the reasons why it is inactive in other *in vitro* systems and in many organs *in vivo*^{3,21}.

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Note added in proof: On 28 June 1979 the FDA banned the use of DES as a growth promoter in cattle and sheep.

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Identical 3' non-coding sequences in five mouse Ig κ chain mRNAs favour a unique C_κ gene

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The constant regions of immunoglobulin κ chains are encoded by a very small but unknown number of genes. The kinetics of nucleic acid reassociation is consistent with up to four C_κ genes per haploid mouse genome^{1–4}, while saturation hybridisation suggests that there are two C_κ genes⁵. The amino acid sequences proposed for seven κ C regions do differ slightly^{6–10}, but at least some of these differences result from sequencing errors¹¹. Determining the nucleotide sequence of the C region of κ mRNAs¹¹ and the adjacent 3' non-coding region should more readily reveal any slight differences reflecting a multiplicity of C_κ genes. Examination of the 3' non-coding region may be particularly useful, as this region is not constrained to encode the largely invariant C_κ amino acid sequence. Sequences reported for the 3' terminal regions of two κ mRNAs, MOPC 21 (refs 11, 12) and MOPC 41A (ref. 13), were identical for the first 59 residues, but the subsequent, tentative, 45 residues proposed for MOPC 41A mRNA differed from the MOPC 21 sequence. It was unclear, however, whether these differences resulted from methodological problems, or genuine sequence divergence. We have therefore re-examined the MOPC 41A 3' terminal sequence in a direct comparison with that of MOPC 21 and also determined 3' terminal sequences of about 100 residues in three other κ mRNAs. We confirm here the sequence presented by Hamlyn *et al.*¹¹ for the MOPC 21 mRNA and show that not only the MOPC 41A mRNA, but also the three other κ mRNAs have the same sequence.

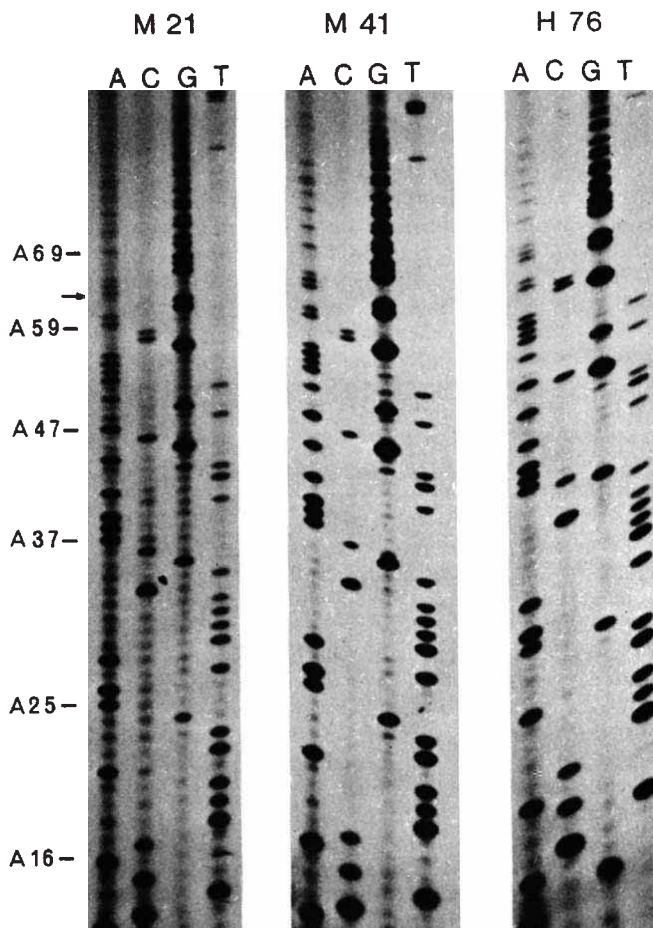


Fig. 1 Polyacrylamide gels used to determine sequences complementary to the 3' termini of MOPC 21, MOPC 41A and HPC 76 κ mRNAs. The oligonucleotide d(pT₁₀-C-A) was used as primer, in a fourfold molar excess over the mRNA templates which were isolated as described previously^{24,25}. cDNA was synthesised in 6- μ l reactions containing 50 mM Tris-Cl pH 8, 50 mM KCl, 8 mM MgCl₂, 10 mM dithiothreitol, 2 μ M [α -³²P]dATP and about 2 units of reverse transcriptase. Dideoxynucleotide triphosphates (P-L Biochemicals) and the corresponding dNTPs were in the following molar ratios: ddATP:dATP, 2.7:1; ddCTP:dCTP, 1.3:1; ddGTP:dGTP, 0.6:1; ddTTP:dTTP, 0.7:1. Incubation was at 39 °C for 15 min, followed by a 'chase' reaction containing 200 μ M of each of the four dNTPs (39 °C, 15 min). Samples were denatured in formamide and loaded onto a 0.35-mm thick 12% polyacrylamide gel²⁶ containing 7 M urea, 90 mM Tris-borate, 2.5 mM EDTA. Electrophoresis was for 2.5 h at 30 mA and autoradiography at -80 °C. The arrow indicates a position at which Hamlyn *et al.*¹¹ have suggested a correction to their previously published sequence for the MOPC 21 κ mRNA¹², the elimination of a C residue; our results confirm this correction.

Sequences were derived by an adaptation^{14,15} of the dideoxynucleotide procedure for sequencing DNA templates¹⁶. Complementary DNA was transcribed using the oligonucleotide d(pT₁₀-C-A)¹² as primer. Figure 1 shows examples of autoradiographs of polyacrylamide gels used to determine sequences complementary to the 3' terminal regions of three κ mRNAs. Clearly, the patterns of bands shown are identical. These gels allowed sequences of about 60–70 nucleotides to be determined. By manipulating the concentrations of the chain-terminating dideoxynucleotides and the time of electrophoresis, sequences of over 100 residues could be obtained. The sequences derived for the five mRNAs examined, and for MOPC 21 (ref. 11), are shown in Fig. 2. At no position did any of the sequences differ distinctly from that of MOPC 21. There were a few positions towards the ends of the sequences where we could not clearly distinguish between two nucleotides, but in each case, one of the choices was that found in the MOPC 21 sequence.

The known 3' terminal sequences in mammalian mRNAs show considerable differences^{11,17–21}. Even between the mRNAs encoding the closely related human and rabbit α -globins and human, rabbit and mouse β -globins there are distinct sequence and length differences within this region^{17–20}. It has been suggested that these regions may not have a precise sequence-dependent function²¹. The apparent identity of the five κ non-coding regions is therefore unlikely to result merely from strong evolutionary selection for a specific sequence, but rather favours the suggestion that there is only one mouse C_{κ} gene. If there were two C_{κ} genes which differed in the region examined and were expressed with equal frequency, the chance that five sequences would be of one type is 1 in 16; similarly, for three C_{κ} genes, the chance would be 1 in 81. Naturally, our results would say little about the possibility of a C_{κ} gene expressed infrequently in myelomas.

Nucleotide sequences reported for a substantial portion of the C region in two κ mRNAs (MOPC 21 (ref. 11) and MOPC 149 (ref. 22)) are identical and contain no 'silent' base changes, providing further evidence in favour of a unique C_{κ} sequence. Similarly, the detailed restriction endonuclease cleavage maps of seven different cloned κ mRNA sequences are identical throughout the C region (N.M.G., E. Webb and J.M.A., in preparation).

It seems remarkable that no differences have been detected in the C_{κ} regions examined, as the plasmacytomas from which they were isolated are of independent neoplastic origin and have been serially transplanted for many years. This suggests that if there is a somatic mechanism for generating V region diversity²³, it must either be directed specifically to the V region genes, or no longer be operational within the terminally differentiated plasma cell.

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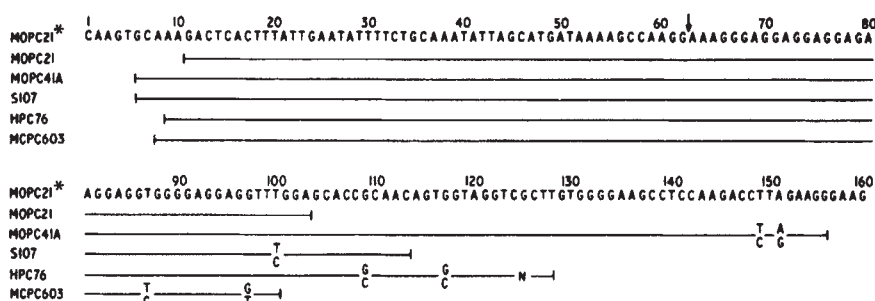


Fig. 2 DNA sequence complementary to the 3' non-coding region of five κ light chain mRNAs. The mRNAs were from the tumours MOPC 41A, MCP603, S107 (reviewed in ref. 27), HPC 76 and from the P3 cell line²⁸ (derived from the tumour MOPC 21). The MOPC 21 κ sequence (*) is taken from the complete sequence of Hamlyn *et al.*¹¹. The solid lines indicate the regions examined in the other mRNAs which had sequences clearly identical to that of MOPC 21. Bands were usually not detected at positions corresponding to the first few residues after the primer, as is often found with this technique. At positions where there was ambiguity the possible residues are given. In the HPC 76 sequence there was one position (N) where a band occurred in the equivalent position in all tracks. The arrow indicates the position at which a C residue had previously been assigned¹² (see Fig. 1).

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A normal cell protein cross-reactive to the major Abelson murine leukaemia virus gene product

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Abelson murine leukaemia virus (A-MuLV) is a replication-defective retrovirus capable of rapid *in vivo* and *in vitro* transformation of bone marrow lymphoid target cells. A-MuLV was isolated from a steroid treated BALB/c mouse inoculated with the replication competent Moloney-MuLV (M-MuLV)^{1–4}. The A-MuLV genome (5.5 kilobases) retains regions of precise homology to M-MuLV at its 5' end (1,320 bases) and 3' end (730 bases). The centre of the A-MuLV genome (3,500 bases) is non-homologous to M-MuLV and presumably represents unique sequences derived from the mouse genome by a recombinational event⁵. The only known protein encoded by the A-MuLV genome is one of MW 120,000, called P120 (refs 6, 7). About one-quarter of this protein is encoded by the 5'-terminal M-MuLV-related sequences and the rest is encoded by the A-MuLV unique sequences. P120 has been found in all cell lines transformed by the strain of A-MuLV that originally derived from ANN-1 cells², including transformed non-producer cells. P120 can be translated *in vitro* using A-MuLV genomic RNA as messenger RNA. P120 is a phosphoprotein but there is no evidence for glycosylation^{6,8,9}. We have used an anti-A-MuLV syngeneic tumour regressor serum with defined specificities to search for cross-reacting normal cellular proteins. We have identified a protein of MW 150,000 (NCP150) in metabolically labelled thymus and other lymphoid organs. NCP150 by absorption and competition analysis is closely related to the unique region of the A-MuLV P120 protein.

Mouse syngeneic tumour regressor antiserum with high reactivity to the A-MuLV unique region of P120 was prepared. After absorption to remove M-MuLV protein reactivity this antiserum contains residual activity for P120 demonstrated by

direct immunoprecipitation and tumour cell surface immunofluorescence⁹. This A-MuLV tumour regressor serum (anti-AbT serum) was used to search by immunoprecipitation for normal cellular proteins.

Explants of mouse lymphoid tissues were labelled for 1 h with ³⁵S-methionine, extracted, immunoprecipitated with anti-AbT serum or normal serum and examined by electrophoresis through an SDS-polyacrylamide gel. A protein of MW 150,000 was precipitated by anti-AbT but not normal serum (Fig. 1); we shall call the protein NCP150 (normal cell protein, 150,000 MW). NCP150 was most prominent in extracts prepared from thymus tissue but smaller amounts were detectable in spleen and bone marrow. Precipitation of NCP150 from thymus has been reproducible; no other protein has reproducibly been reactive with anti-AbT serum but not normal serum. NCP150 has been found in BALB/c, C57BL/6, C57L and SJL mouse strains. No additional proteins were found if ³H-leucine or ¹⁴C-labelled mixed amino acids were used to label thymus tissue (data not shown).

The reactivity of our anti-AbT serum with NCP150 could not be blocked by unlabelled M-MuLV virion proteins (100 µg ml⁻¹) (included in all samples in Fig. 1) nor could any sera specifically reactive with M-MuLV proteins encoded in the *gag*, *pol* or *env* gene regions¹⁰ precipitate NCP150.

To examine more critically whether NCP150 was being precipitated by antibody reactive to the unique A-MuLV determinants of the P120 molecule, we performed competition and absorption tests. Extracts of C57BL/6 thymus labelled with ³⁵S-methionine were the source of NCP150, and extracts of 2M3 cells—an A-MuLV-transformed lymphoid cell line—were used as a comparison source of labelled P120. Anti-AbT serum in the presence of excess unlabelled M-MuLV virion proteins was used to precipitate all experimental samples and unlabelled cell extracts were used as competitor.

Normal mouse serum precipitated no NCP150 or P120 (Fig. 2, lanes *a*) and anti-AbT serum precipitated readily detectable bands of NCP150 and P120 (Fig. 2, lanes *b*). The amounts of anti-AbT serum used had been titrated to be limiting in the precipitation. When increasing amounts of unlabelled extracts of an A-MuLV-transformed non-producer NIH/3T3 cell line were included, immunoprecipitation of both NCP150 and P120 was blocked (Fig. 2, lanes *c–e*). Equivalent amounts of extract from a Moloney sarcoma virus transformed non-producer NIH/3T3 cell line did not block the specific precipitations (Fig. 2, lanes *f–h*). Extracts of NIH/3T3 cells infected with the spleen focus-forming component of the Friend mouse leukaemia virus also failed to block precipitation (data not shown). Thus competition experiments show that all of the reactivity of anti-AbT serum with NCP150 can be blocked by extracts containing P120 (A-MuLV transformed cells) and not by extracts of transformed cells that lack P120 (Moloney sarcoma virus transformed cells).

As another specificity test, we pre-absorbed anti-AbT serum with intact, viable A-MuLV-transformed non-producer NIH/3T3 cells to remove reactivity to the P120 determinants expressed on the cell surface. Such absorptions have been shown⁹ to remove the ability of anti-AbT serum to stain the cell membrane of A-MuLV transformed cells; it also markedly reduced the ability of the serum to immunoprecipitate P120. Figure 2, lanes *i*, shows that precipitation of NCP150 and P120 with unabsorbed control serum was evident but lanes *j* show a markedly reduced precipitation with the serum preabsorbed by live cells. Thus, for selected batches of anti-AbT serum, most of the reactivity is with cell surface-exposed regions of P120 and these regions are also those by which NCP150 is precipitated. Whether these regions of NCP150 are cell surface-exposed remains to be determined.

These results demonstrate that NCP150 and the unique region of P120 share common antigenic determinants. The precise arrangement of these antigenic sites and molecular extent of homology between NCP150 and P120 cannot be determined using these techniques. Further work involving

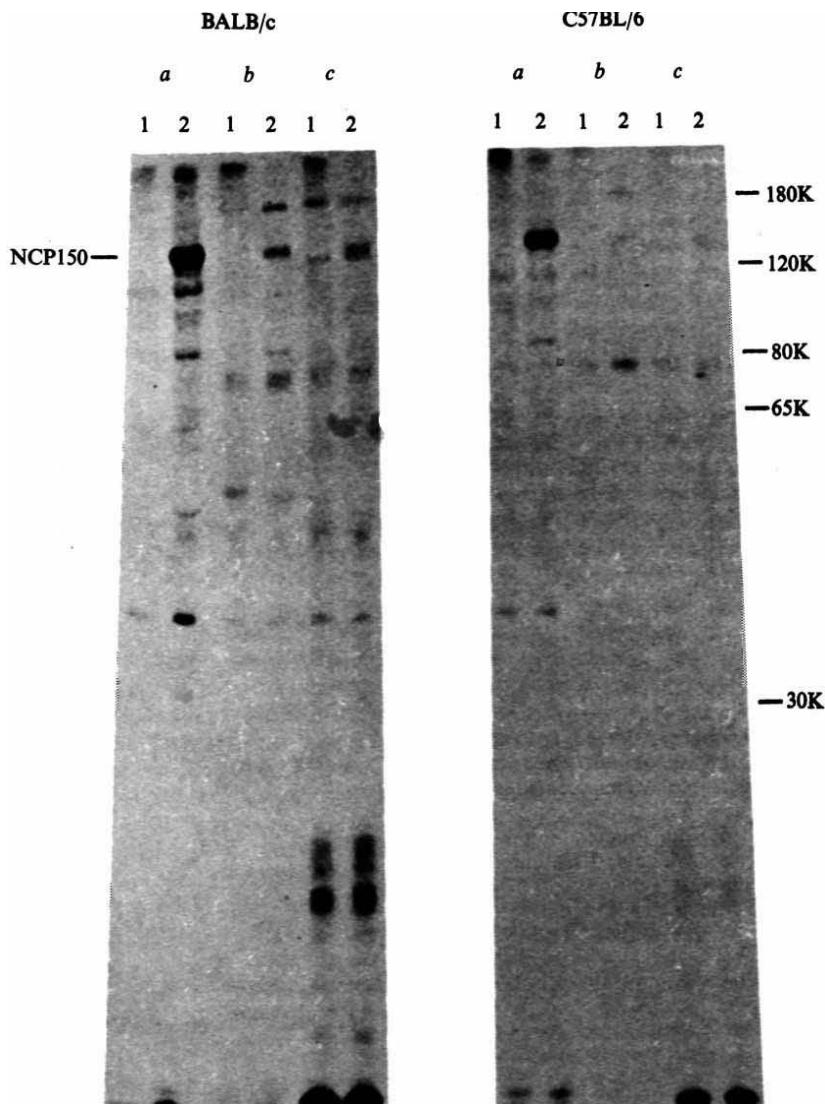


Fig. 1 Immunoprecipitation of a normal cellular protein with anti-Abelson MuLV tumour regressor serum. *In vitro* explants prepared as single cell suspensions (2×10^7 nucleated cells) of thymus (a), spleen (b) or bone marrow (c) from normal 5-week-old BALB/c or C57BL/6 mice were labelled with 100 μ Ci of 35 S-methionine (NEN) in 2 ml of methionine free media with 10% dialysed fetal calf serum for 1 h at 37 °C. Cells were pelleted, extracted and clarified for immunoprecipitation as previously described⁶ at 10^7 cells per ml with the inclusion of the protease inhibitor phenylmethylsulphonylfluoride at 2 mM. Aliquots of 1 ml were reacted (16 h at 4 °C) with 5 μ l of either normal mouse serum (lane 1) or anti-Abelson MuLV syngeneic tumour regressor serum (anti-AbT, lane 2). All tubes contained 100 μ g ml⁻¹ unlabelled virion proteins of Moloney MuLV. Immunoprecipitates were collected with *Staphylococcus aureus* and analysed on a SDS-10% polyacrylamide gel developed by fluorography as previously described¹⁰. The migration position of molecular weight standards given is shown on the right hand side.

formal peptide map comparison will be required to answer such questions.

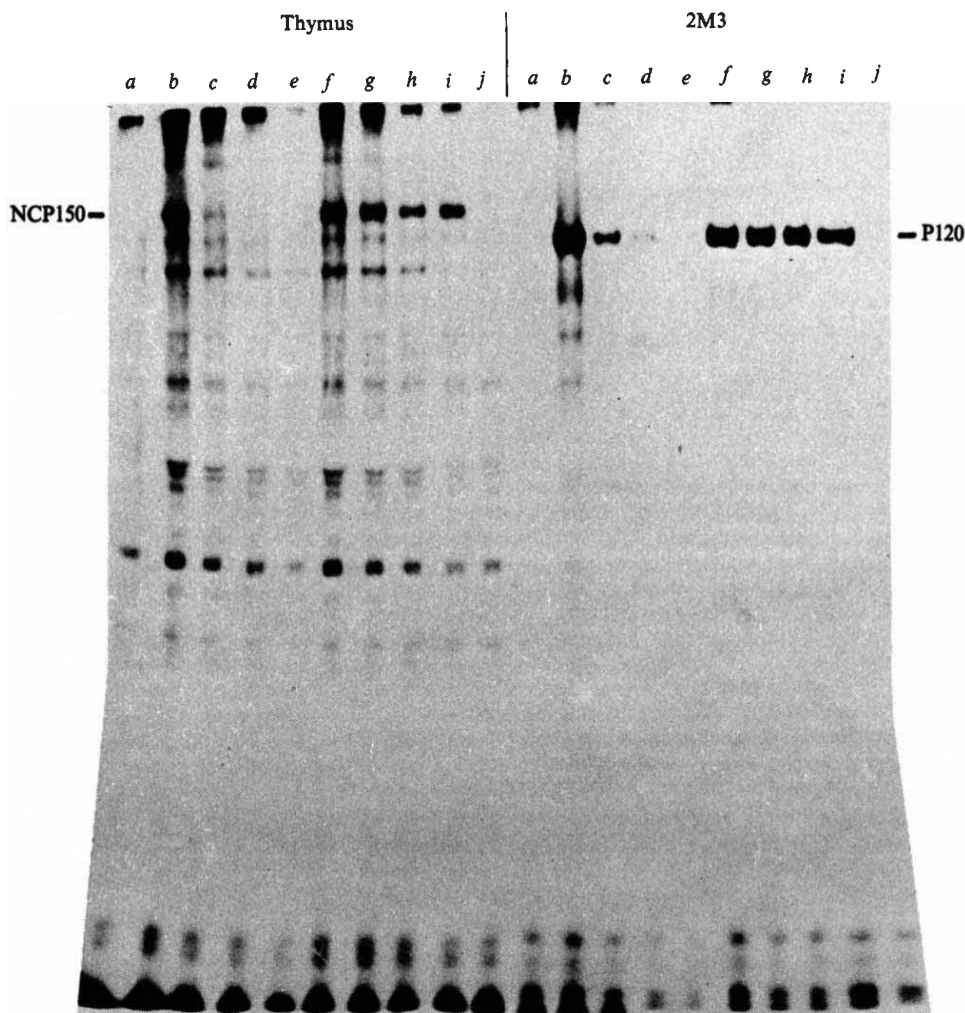
The complete spectrum of cell types that contain NCP150 and the relative concentrations of the protein in these cells have yet to be ascertained. Using a 1-h pulse with 35 S-methionine, thymus certainly contained more labelled NCP150 than spleen or bone marrow (Fig. 1) but unknowns of turnover time and cellular heterogeneity make statements about relative concentrations of NCP150 premature. The detection of NCP150 in spleen and marrow is near the borderline of our present techniques. Using these techniques, cultured fibroblasts from a variety of mouse strains and liver cells have shown no evidence of NCP150. It seems likely from the present results that NCP150 shows a significant variation in concentration as a function of cell type. It also seems from comparisons of band intensities on autoradiograms that the amount of P120 is 40- to 100-fold higher per cell in A-MuLV-transformed cells than the amount of NCP150 in lymphoid tissues. Various cultured murine thymic leukaemia cells have been surveyed and while all showed evidence of NCP150, none was especially high (data not shown).

Work in the avian sarcoma virus system (ASV) has demonstrated the utility of tumour regressor antisera for identifying the normal cellular protein analogue of the transforming gene product, pp60^{src}. In the ASV system the normal cellular pp60 and viral pp60^{src} proteins were very close in molecular weight. The normal cellular pp60 was found in fibroblast tissues of a variety of avian and other vertebrate species but the amount of

normal cellular pp60 was 10- to 100-fold less than pp60^{src} in ASV transformed cells^{11,12}. The ASV and A-MuLV systems appear similar in that sera that recognise the transforming protein—or the putative transforming protein in the case of A-MuLV—also recognise a normal cellular constituent. The systems differ, however, in that ASV appears to contain a total cellular gene with no added viral determinants while A-MuLV has only part of a cellular gene (about 90,000 of a total MW of 150,000) attached to amino acid sequences of normal M-MuLV proteins. The systems also differ in that the messenger RNA and presumably the protein representing the normal *src* equivalent is present at a low but consistent level in all cells and tissues examined¹³ but NCP150 is more variable in occurrence. Both normal cell proteins occur at concentrations much lower than their counterparts in virally transformed cells.

Our results should be contrasted with those of Risser *et al.*¹⁴. They have prepared a similar mouse anti-A-MuLV syngeneic regressor serum and used it to identify an A-MuLV related tumour cell surface antigen by cytotoxicity. No biochemical determination of the nature of this antigen has been reported. Using lymphoid cells of normal mice in absorption tests, they reported that a cross-reacting antigen can be detected on the surface of bone marrow and spleen cells but not thymus from BALB/c and closely related strains. Unrelated strains, like C57BL/6, were negative for this normal cellular antigen. Our results with NCP150 show a different tissue distribution from that described by Risser *et al.* and we do not find any evidence of allotypy in our serum. Whether the differences between the

Fig. 2 Competition and absorption analysis of NCP150 and P120. Labelled cell extracts of C57BL/6 thymus and 2M3 cells (an A-MuLV *in vitro* derived clonal lymphoid cell line)⁶ were prepared and analysed as in Fig. 1. A final volume of 1 ml for each cell extract of thymus (10^7 cells) or 2M3 (5×10^5 cells) was used for each tube. All tubes contained $100 \mu\text{g ml}^{-1}$ unlabelled Moloney MuLV virion proteins. Lane a, normal mouse serum (5 μl). Lane b, anti-AbT (5 μl). Lanes c, d and e, anti-AbT (5 μl) plus unlabelled extract of 2×10^6 , 4×10^6 or 8×10^6 A-MuLV transformed non-producer NIH/3T3 cells. Lanes f, g, h, anti-AbT (5 μl) plus unlabelled extract of 2×10^6 , 4×10^6 , or 8×10^6 Moloney sarcoma virus transformed non-producer NIH 3T3 cells respectively. Lane i, 1:10 diluted anti-AbT (50 μl) control-mock absorbed. Lane j, 1:10 anti-AbT (50 μl) pre-absorbed with viable A-MuLV transformed non-producer NIH 3T3 cells (2×10^8 cells per ml diluted serum—3 cycles each 45 min; 0°C). Immune precipitates were prepared and analysed as in Fig. 1.



results imply that we are studying different proteins remains unclear. One possibility is that Risser *et al.* were detecting NCP150 but that the synthetic rate (as monitored by immunoprecipitation) is controlled separately from expression at the cell surface (as monitored by cytotoxicity absorption).

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***Clostridium botulinum* can grow and form toxin at pH values lower than 4.6**

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It is generally accepted¹ that in *Clostridium botulinum* both growth and toxin formation are completely inhibited at pH values below 4.6. This critical pH value has been confirmed by many investigators using food as substrate²⁻⁵ or culture media^{3,6-8}. Occasionally⁹⁻¹² growth of *C. botulinum* and toxin formation at pH values lower than 4.6 have been reported. In these cases the authors ascribed the unexpected outgrowth and toxin formation to local pH differences in inhomogeneous media and growth of *C. botulinum* before pH equilibration, or to the fact that fungi created microenvironments within or adjacent to the mycelial mat, where the pH was higher than 4.6 as was demonstrated by Odlaug and Pflug^{11,12}. We show here that the general assumption that *C. botulinum* does not grow below pH 4.6 is incorrect. We have observed that growth and toxin formation by *C. botulinum* can take place in homogeneous protein rich substrates (containing 3% or more soya or milk protein) at pH values lower than 4.6.

Table 1 Influence of pH, type of acid, and protein content on the behaviour of *C. botulinum* type A and B, if inoculated together with *Bacillus* spores in substrates based on protein enriched soya extracts

Acid	Initial pH	Protein content (% w/w)		Incubation (weeks at 30 °C)							
				1	2	4	6	8	10	12	14
HCl	4.0	5.5	(1)	+-	--	--	++	+-	+-	--	+-
			(2)	--	--	--	++	+-	+-	--	+-
			(3)	θ	θ	θ	II	I	I	θ	I
HCl	4.2	5.5	(1)	--	+-	++	+-	+-	--	++	+-
			(2)	--	--	++	+-	+-	--	+-	+-
			(3)	θ	θ	II	I	I	θ	I	I
HCl	4.4	5.5	(1)	++	++	++					
			(2)	++	++	++			NT		
			(3)	I	II	II					
HCl	4.4	3.0	(1)	+-	+-	--	+-	--	--		
			(2)	+-	+-	--	+-	--	--		NT
			(3)	I	I	θ	I	θ	θ		
HCl	4.4	0.6	(1)	--	--	--	+-	++	++	--	++
			(2)	--	--	--	--	--	--	--	--
			(3)	θ	θ	θ	θ	θ	θ	θ	θ
Citric acid	4.4	5.5	(1)	+-	+-	++	++	++	+-	++	++
			(2)	--	--	+-	++	++	+-	++	++
			(3)	θ	θ	I	II	II	I	II	II
Lactic acid	4.4	5.5	(1)	--	--	--	--	--	--	+-	--
			(2)	--	--	--	--	--	--	+-	--
			(3)	θ	θ	θ	θ	θ	θ	I	θ
Acetic acid	4.4	5.5	(1)	--	--	--	--	--	--	--	+-
			(2)	--	--	--	--	--	--	--	+-
			(3)	θ	θ	θ	θ	θ	θ	θ	I

(1) Initial inoculum 100 bacilli per ml substrate. At least hundred-fold growth of *Bacillus* in one sample (+) or two samples (++). Less than hundred-fold growth of *Bacillus* in one sample (-) or two samples (--).

(2) Initial inoculum 1,000 *C. botulinum* per ml substrate. At least 10-fold growth of *C. botulinum* in one sample (+) or two samples (++). Less than 10-fold growth of *C. botulinum* in one sample (-) or two samples (--).

(3) θ, Two samples tested for toxin, no sample toxic; I, 1/2 samples toxic; II, 2/2 toxic.

NT, Not tested.

Media in screwcap bottles containing different levels of soya or milk proteins, in which the pH was adjusted with various acids (Table 1), were inoculated with either a mixture of spores of *C. botulinum* type A strain 62A, VI₁ and ZK₃ and *C. botulinum* type B strain B6 and 2345, alone or together with a mixture of spores of *Bacillus subtilis* and *B. licheniformis* isolated from soya concentrates. Details are given in Table 1.

The inoculated media were filled into screwcap bottles which were subsequently heated for 5 min at 100 °C. After cooling, the headspace in each bottle was filled with liquid paraffin. Duplicate bottles were examined at various intervals during incubation at 30 °C for growth, pH value and toxin; the presence of toxin was tested by intraperitoneal injection of mice.

The pH value did not increase from the initial values by more than 0.1 pH unit during incubation. Growth and toxin formation by *C. botulinum* took place at a pH value as low as 4.0 in the presence of the bacilli if the pH was adjusted with hydrochloric acid. Growth of the bacilli removed residual oxygen and lowered the redox potential, thus creating more favourable conditions for the growth of *C. botulinum*. When we compared the inhibitory action of hydrochloric, citric, lactic and acetic acids at pH 4.4, growth and toxin formation decreased in that order (Table 1).

The level of protein in the media also affected the ability to grow at low pH values—at pH 4.4 a minimum protein level around 3% was necessary for growth and toxin formation.

In further experiments with 5.5% soya protein J. S. Crowther (personal communication) observed that in anaerobic conditions spores of *C. botulinum* are able to outgrow and form toxin at pH 4.2 (if hydrochloric acid was used as acidulant) in the absence of other microorganisms. However the results at this pH were not always repeatable.

So far we have not found *C. botulinum* to grow in a wide range of actual foods with pH value lower than 4.6, but the assumption that *C. botulinum* does not grow below pH 4.6 has been shown to be incorrect, and the understanding of conditions in which growth may occur, requires further study.

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Superoxide involvement in the bactericidal effects of negative air ions on *Staphylococcus albus*

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The physical nature of small air ions is well established and it is recognised that they can produce a variety of biological effects¹. However, in only a few instances have any underlying biochemical changes been detected²⁻⁴. Theoretically, one can consider the hydrated superoxide radical anion (O_2^-) (H_2O)_n with $n \approx 4-8$ as a likely candidate for a biologically active species of negative air ion⁵. The chemical and biological reactivity of superoxide is high⁶ and includes a leading role in bacterial killing caused by radiation^{7,8}, in which superoxide dismutase (SOD), an enzyme that catalyses the reaction:



protected markedly. Other studies have also demonstrated the bactericidal effect of O_2^- (refs 9-11). Inasmuch as the bactericidal action of small negative air ions has been repeatedly confirmed, we decided to test for the involvement of O_2^- in this phenomenon by evaluating the protective effect of SOD. Our results show strong O_2^- involvement in negative air ion bacterial kill.

Figure 1 illustrates the apparatus used in our experiments. To ensure that all negative ions went through the solution to the ground wire, we insulated the ground wire using heat shrink tubing and Teflon tubing to a point well below the solution's surface. A Modulon corona discharge type negative ion generator provided the ionising potential; to maintain a uniform current we adjusted the ionising potential using separate variable transformers on the a.c. lines of each generator.

Inoculated flasks, containing *Staphylococcus albus* at $\sim 10^6$ cells per ml in 40 ml of sterile 3.75 mM NaCl with 1.25 mM KPi, pH 7.8 buffer, were maintained at $22 \pm 1^\circ C$ in a waterbath for the duration of the experiment. A vacuum pump with airflow regulator exhausted air from the flasks at a rate of approximately 500 ml per min. Aliquots of 1 ml were removed hourly, serially diluted, and then plated as previously described¹².

We isolated superoxide dismutase from bovine liver using the procedure of McCord and Fridovich¹³. This yielded pure enzyme of high specific activity ($\sim 3,500$ U ml⁻¹). Catalase (Boehringer Mannheim) was extensively dialysed before use.

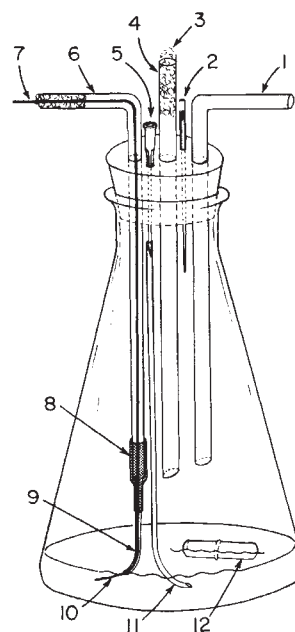


Fig. 1 1, Air exhaust tube. 2, Ion discharge needle. 3, Cotton plug. 4, Air inlet tube. 5, 18 gauge hypodermic needle. 6, Ground wire tube. 7, Ground wire. 8, Heat shrink tubing. 9, 1-mm Teflon insulating tube. 10, Bare platinum tip. 11, 1-mm Teflon sample tube. 12, Magnetic stirring rod. The apparatus was constructed in a 500 ml Erlenmeyer flask fitted with a no. 10 rubber stopper.

Enzymes were sterilised by passage through a Millipore filter immediately before addition to the bacterial inoculum.

Table 1 shows that the exposure of bacteria to negative air ions caused a substantial loss in viability in as short a period as 2 h, with a total loss of viability seen after 5 h of ion treatment. Control samples, incubated in identical conditions but with no negative ionisation, retained full viability after 5 h. Addition of $10 \mu g$ ml⁻¹ SOD protected bacteria completely against negative ion killing while catalase or denatured SOD had no significant protective effect. The lack of effect of catalase in this system appears in marked contrast to that seen in most systems which involve O_2^- toxicity. For example, both O_2^- and H_2O_2 were required for lipid peroxidation or erythrocyte lysis¹⁴ where their toxicity manifests through their interaction to produce hydroxyl radical and singlet oxygen. In the case of negative air ion bacterial kill the toxicity of O_2^- appears H_2O_2 independent. A possible mechanism might involve O_2^- acting as a nucleophile on the phospholipid bilayer, causing a de-esterification of fatty acids¹⁵. This could lead to an increase in surface charge and a weakening of the membrane, which under hypotonic conditions might lead to cell lysis and death.

The experiments reported here support the concept that the negative air ions responsible for bacterial death consist solely or in part of hydrated superoxide anions. Paradoxically, while negative air ions have a reputation as a rather beneficial species¹, superoxide radical anion apparently acts as one of the

Table 1 Bacterial kill by negative air ions (% viability)

Conditions	Incubation time (h)					
	0	1	2	3	4	5
No (-) ions (control)	100	123 $\pm$ 3.4	115 $\pm$ 22.5	112.5 $\pm$ 1.0	111.6 $\pm$ 22.3	105.6 $\pm$ 11.0
(-) Ions	100	116 $\pm$ 3.5	72.3 $\pm$ 9.9	29.2 $\pm$ 24.9	2.5 $\pm$ 2.3	0
(-) Ions + $10 \mu g$ ml ⁻¹ SOD	100	129.0 $\pm$ 1.0	106.2 $\pm$ 18.8	134.0 $\pm$ 13.0	109.0 $\pm$ 25.1	104.8 $\pm$ 14.2
(-) Ions + $10 \mu g$ ml ⁻¹ (denatured autoclaved SOD)	100	87.9 $\pm$ 7.1	70.0 $\pm$ 18.1	27.1 $\pm$ 1.1	5.5 $\pm$ 1.4	0
(-) Ions + $10 \mu g$ ml ⁻¹ catalase	100	105.2 $\pm$ 23.8	56.7 $\pm$ 10.4	21.5 $\pm$ 4.4	11.9 $\pm$ 3.4	1.0 $\pm$ 1.0

Negative ion flux was maintained at approximately $5 \mu A$ in all experiments. $\pm$ Figures indicate the range between duplicate experiments.

most toxic species produced in aerobic metabolism⁶. Perhaps the resolution of this paradox lies in the relative amount of O_2^- required for an effect. Negative air ions can cause physiological effects at extremely low levels, with clean country air having a negative ion concentration of only about 10^3 cm^{-3} , an amount which more closely parallels that of pheromones than of chemical reactants. In our system we have greatly amplified the flux of negative ions to a level which could produce, theoretically at least, 0.3 mmol O_2^- per h, which we channelled through a total of 40 ml of solution. Even so, *in vitro* systems testing for O_2^- reactivity have invoked a flux hundreds of times greater. Thus the effects of superoxide as a negative air ion although dependent on its chemical reactivity may be greatly amplified by a biological sensitivity in the hormonal range. Our tentative identification of $(O_2^-)(H_2O)_n$ as the negative air ions responsible for killing micro-organisms should provide a more solid base for investigating the effects and mechanisms of negative air ion effects in other biological systems.

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Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion

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We have previously regenerated tobacco plants from fused protoplasts isolated from two varieties of *Nicotiana tabacum*. The parent cells had distinct morphological nuclear and cytoplasmic markers, enabling us to recognise, among the whole regenerated plants, those with a single parental nucleus and a hybrid cytoplasm produced by the mixing of the two parental cytoplasm¹. Genetic analysis of their progeny has confirmed that the phenotypes of these cytoplasmic hybrids (or cybrids) are stable and maternally inherited. Analysis with restriction endonuclease has shown that only one or the other parental chloroplast DNA is present in the progeny of the cybrids². We report here, however, that the mitochondrial (mt) DNAs of cybrids are different from those of the parents and from the mixture of the two. The new DNAs result from mitochondrial recombination.

Cytoplasmic hybrids were obtained from fusion between protoplasts of *N. tabacum* var. Xanthi (Xf) which has normal fertile flowers and petiolated leaves, and *N. tabacum* var.

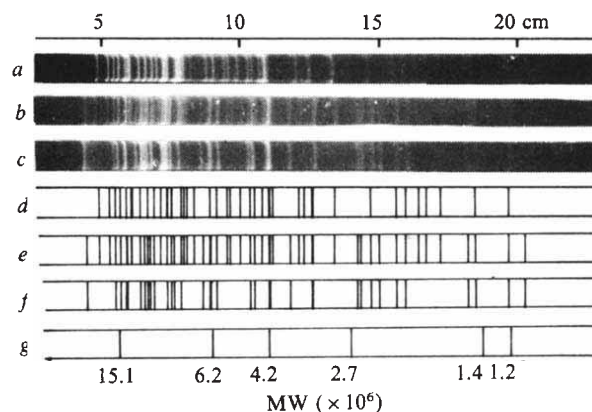


Fig. 1 Agarose slab gel electrophoresis of *SalI* digests of mtDNAs from: a, Ts or *N. tabacum* var. Techne (*debneyi* cytoplasm); c, Xf or *N. tabacum* var. Xanthi (*tabacum* cytoplasm); b, mixture of Ts and Xf. These diagrams are schematically represented on d, (Ts); e, (Ts + Xf); f, (Xf). *HindIII* fragments of λ DNA (g) were used as standards for molecular weight determinations. Mitochondria were isolated as previously described³ with the following modifications. Green leaves were used because etiolated material cannot be obtained with tobacco. DNase-treated organelles were purified by centrifugation on two layers of 30 and 60% sucrose. mtDNA was extracted as described for cp-DNA^{6,7} and purified by linear CsCl-ethidium bromide gradient centrifugation for 2 d at 32,000 r.p.m. in a SW41 rotor. This gives cleaner mtDNA bands than does the two-step procedure used before. *SalI* enzyme was prepared according to R. J. Roberts (unpublished). Experimental conditions for mtDNA digestion, agarose gel electrophoresis and UV fluorescence photography were as described^{5,7}.

Techne (Ts), which has abnormal flowers with cytoplasmic male sterility, producing no pollen. The Ts variety, obtained from the interspecific cross *N. debneyi* $\times$ *N. tabacum*, has the cytoplasm of the former and the nucleus of the latter. The resulting nucleocytoplasmic interaction is responsible for the male sterility. Ts has sessile leaves, and in our experimental system leaf shape is the nuclear marker and flower shape the cytoplasmic marker. Details of the parents and cybrids are shown in Table 1.

Restriction endonuclease was used to analyse mtDNAs extracted from the progeny of nine cybrids (six Ti and three Xi) which represent all the variability of the new i phenotypes (see legend to Table 2). Mitochondria and mtDNAs were isolated as described in Fig. 1 legend.

Figure 1 shows the *SalI* restriction patterns for mtDNA from the Ts (a) and Xf (c) parents showing 38 and 31 fluorescent bands, respectively. Of these, 20 appear to be common to *N. debneyi* and *N. tabacum* mtDNAs, 18 specific to Ts (*N. debneyi*) mtDNA and 11 specific to Xf (*N. tabacum*) mtDNA, as shown by comparison of Ts, Xf and Ts + Xf schematic diagrams (Fig. 1). Consequently, the diagram corresponding to a mixture of the two parent mtDNAs has 49 bands (Table 2). We found that the two varieties of *N. tabacum*, Xf and Tf (fertile Techne containing the *N. tabacum* cytoplasm), had identical mtDNAs. Figures 2 and 3 show the *SalI* restriction patterns of mtDNAs

Table 1 Genotypes and phenotypes of parent and cybrid plants

Name	Genotypes		Phenotypes		
	Nucleus	Cytoplasm	Leaf	Flower	
Parents	{ Ts { { Xf {	<i>N. tabacum</i> var. Techne	<i>N. debneyi</i>	Sessile	Abnormal; male sterile type s
		<i>N. tabacum</i> var. Xanthi	<i>N. tabacum</i>	Petiolated	Normal; male fertile type f
Cybrids	{ Ti { { Xi {	<i>N. tabacum</i> var. Techne	hybrid <i>N. debneyi</i> × <i>N. tabacum</i>	Sessile	Intermediate between those of the parents
		<i>N. tabacum</i> var. Xanthi	hybrid <i>N. debneyi</i> × <i>N. tabacum</i>	Petiolated	

Table 2 *SalI* digests of mtDNAs

Plants	Phenotype	Bands					MW* ($\times 10^6$)
		Common	<i>debneyi</i>	<i>tabacum</i>	New	Total	
Ti	Xf, Tf	20	—	11	—	31	206
	Ts, Tsp	20	18	—	—	38	256
	T ₁₆₁	20	8	7	3	38	293
	T ₄₇	18	9	7	1	35	269
	T ₂₇	19	12	7	1	39	264
	T ₁	17	12	5	4	38	260
	T ₇₅	17	15	3	2	37	267
	T ₁₃	20	18	1	—	39	259
	Xi	20	16	3	1	40	248
	X ₁	19	17	3	2	41	296
Xi	X ₇	20	17	1	1	39	290

Xf, Tf: Varieties with *tabacum* cytoplasm, normal and fertile flowers. Ts: Variety with *debneyi* cytoplasm, abnormal and male sterile flowers with split corolla, short stamen filament ended by a stigmathe. Tsp: Control plant: plant regenerated from Ts protoplast with abnormal and male sterile flowers identical to Ts parent ones. Ti and Xi: cybrid plants are defined in Table 1. i Types: I, normal corolla, filament shorter than the normal, anther with some pollen: fertile. II, slightly nicked petals, long filament with flat anther: the most often sterile. III, nicked petals, long filament with distorted anther: sterile. IV, split corolla, short filament ended by white outgrowths: sterile.

* Approximate molecular weights obtained as described in the text.

isolated from the progeny of the regenerated cybrids Ti and Xi, obtained by crossing, respectively, female Ti $\times$ male Tf and female Xi $\times$ male Xf. Each of the nine cybrids in Table 2 contained a unique mtDNA distinguishable from both parent mtDNAs and from a mixture of the two. However, the cybrid patterns always contained mtDNA fragments belonging to both parents, and also, in all but one, new bands appeared. The number of bands on the cybrid diagrams varied from 35 to 41, confirming that these mtDNAs were not products of the addition of two parental mtDNAs.

The complexity of the restriction patterns precludes accurate determination of molecular weight. Nevertheless, estimates obtained by summing the molecular weights of the bands (band multiplicities are not taken into account and it is difficult to determine precisely the size of the larger fragments) show that the cybrid mtDNAs more closely resemble the Ts mtDNA than the Xf mtDNA or the mixture of parental mtDNAs (Table 2). Table 2 also shows for each cybrid mtDNA the distribution of the specific and common parental bands. There is clearly a correlation between flower shape and male sterility or fertility and the ratio of the specific *N. debneyi* and *N. tabacum* bands: an increase in the degree of sterility in the different i phenotypes of the cybrids corresponds to an increase in the number of *N. debneyi* specific fragments.

The fate of mtDNA after the initial fusion of *N. debneyi* and *N. tabacum* cytoplasm is strikingly different from that of

chloroplast DNA in our cybrid plants². Our results, together with previous observations of maternal inheritance and stability of floral markers, constitute evidence that new cytoplasmic characters characterised by specific mtDNAs arise from protoplast fusion. Consequently, flower morphology and male sterility in tobacco must be controlled by nucleo-mitochondrial interaction. The presence of new bands in the cybrid mtDNA restriction patterns is evidence of mitochondrial recombination in somatic hybrid cells: the simultaneous presence, in the same cell, of *N. tabacum* and *N. debneyi* mitochondrial genomes is followed by mtDNA recombination because new molecular species are observed. Fonty *et al.*³ have used restriction enzymes to analyse the mitochondrial genome of yeast cells and have provided the first evidence for physical recombination of mtDNA. They have also indicated that this event is frequent in crosses of wild type yeast cells.

The advantages of somatic cell hybridisation for studying non-chromosomal inheritance in higher plants were first pointed out by Gleba⁴. Unfortunately our knowledge of the mitochondrial genetics of higher plants is still limited by the scarcity of biochemical and genetic markers (namely resistance markers). The cytoplasmic parasexual hybrids of *N. tabacum* analysed here are identified by their floral phenotypes and also by specific mtDNAs arising from recombinations between different mtDNAs. The cytoplasmic male sterility in tobacco involves the mitochondria and can be transferred by protoplast fusion from one variety to another. These results emphasise the potential of somatic cell fusion for studying mitochondrial genetics and for increasing the cytoplasmic variability in higher plants.

We thank Dr F. Quetier for *SalI* endonuclease and helpful discussions.

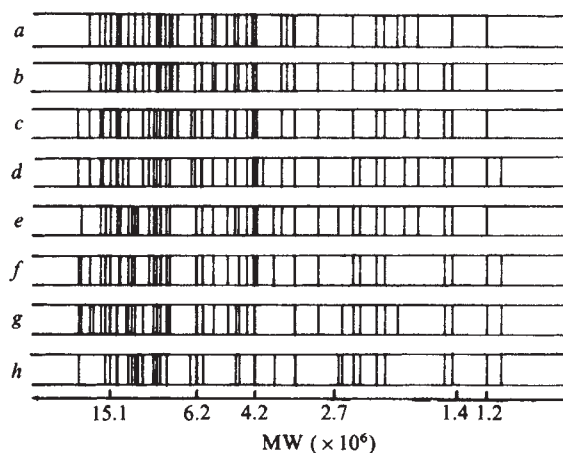


Fig. 2 *SalI* band patterns on 0.7% agarose gel shown by the mtDNAs from parents: a, Ts; h, Xf, and from some Ti cybrids of first generation: b, T₁₃; c, T₇₅; d, T₁; e, T₂₇; f, T₄₇; g, T₁₆₁. Ti plants were characterised by the nuclear genome of the Ts parent (Ti plants and Ts parent presented identical leaf shape) and by hybrid cytoplasm (the flower shapes of the Ti plants were intermediate between the two parental ones). Isolation and analysis of the mtDNAs were as described in Fig. 1.

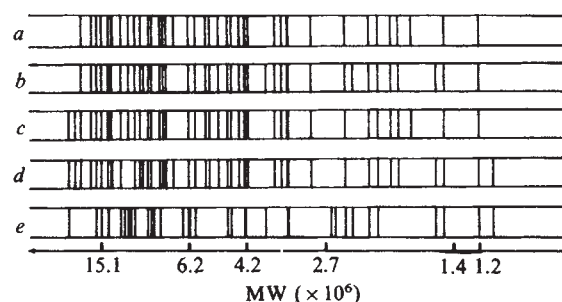


Fig. 3 *SalI* band patterns on 0.7% agarose gel shown by the mtDNAs from parents: a, Ts; e, Xf, and from some Xi cybrids of first generation: b, X₆; c, X₇; d, X₁. Xi plants were characterised by the nuclear genome of the Xf parent (Xi plants and Xf parent presented identical leaf shape) and by hybrid cytoplasm (the flower shapes of the Xi plants were intermediate between the two parental ones). Experimental procedure as for Fig. 1.

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Pretreatment with acetylaminofluorene enhances the repair of O^6 -methylguanine in DNA

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Epidemiological and experimental evidence suggests that the interplay of environmental factors may be particularly relevant to the induction of cancer in man^{1,2}. We have investigated the possible interrelationships, at the level of DNA repair, of two chemically different hepatocarcinogens administered to rats. Animals were pretreated for several weeks by inclusion of 2-acetylaminofluorene (AAF) in the diet and a study was made of the capacity of liver to repair lesions subsequently introduced into DNA by a pulse of dimethylnitrosamine (DMN). Of particular interest was the repair of adducts at the O^6 position of guanine as this product has been implicated as a critical reaction site for carcinogenesis by the monofunctional alkylating agents^{3,4}. We show here that the capacity of liver to repair O^6 -methylguanine in DNA is enhanced by prolonged exposure of rats to the chemically unrelated agent, AAF.

Male Wistar rats, 7–8 weeks old and weighing ~200 g, were given (*ad libitum*) standard laboratory diet containing 0.06% AAF for three weeks and were then transferred to normal diet for the fourth week. Three such feeding cycles were administered and DMN was given at the end of the final week (Fig. 1). At this stage the animals were 19–20 weeks old and weighed ~270 g, much lower than the weight (400 g) of a normal 20-week-old colony-bred rat. This weight difference was apparently due to the lower food consumption during exposure to the carcinogen diet, as a group of animals kept throughout the 12 weeks on a schedule of equivalent daily food intake per rat also reached a body weight of about 270 g. All animals, AAF-pretreated and controls, received a single intraperitoneal (i.p.) injection of (^{14}C -methyl)-labelled DMN and were later killed at various intervals. Liver DNA was isolated and analysed as described (see Table 1 legend) to determine the amounts of O^6 -methylguanine and 7-methylguanine as a proportion of the parent nucleotides. As evidence suggests that 7-methylguanine is lost in a constant and predictable manner from the liver DNA of normal rats by chemical depurination reactions⁵ the amounts of O^6 -methylguanine were expressed as a ratio of the 7-methylguanine present in the same DNA to provide an estimate of the relative persistence of this O -methylated product. This ratio also provides a convenient internal standard to allow for any effects of toxicity that may lead to cell lysis, which would affect the loss of all DNA products in the same way.

Following DMN treatment at 5.5 mg kg⁻¹, the amounts of O^6 -methylguanine and 7-methylguanine present in liver DNA of the control rats kept on an equivalent daily food intake were determined (Table 1). The results are similar to those obtained for weight-matched controls and lie within the expected range⁶ both with regard to the amount of base formed and the ratio of O^6 -methyl- to 7-methylguanine (compare with Figs 2 and 3). Data from these weight-matched controls have been used for comparison with AAF-pretreated animals. Results presented here are for AAF-pretreated rats with livers of normal

Table 1 Amounts of O^6 -methylguanine and 7-methylguanine in liver DNA of control rats

DNA reaction product	Time after DMN treatment		
	5 h	8 h	24 h
7-Methylguanine*	412	378	275
O^6 -Methylguanine*	39.8	36.9	14.4
O^6 -Methyl/7-Methylguanine	0.097	0.098	0.053

Control rats, having received daily food intake equivalent to that of the AAF-pretreated animals, (see text) were treated with a single (i.p.) injection of DMN (5.5 mg kg⁻¹, 5 mCi mmol⁻¹). The livers of 3 rats were pooled for each time point, DNA was extracted by a modified phenol procedure¹⁸ and hydrolysed in 0.1 M HCl (70 °C, 30 min). The authentic marker compounds 7-methylguanine, 3-methyladenine and O^6 -methylguanine were added and the pH of the hydrolysate adjusted to 2.8. Purine bases were separated by chromatography¹⁹ on columns of (1 × 100 cm) Sephadex G-10 eluted with 0.05 M ammonium formate—0.2% (w/w) sodium azide, pH 6.75 at a flow rate of 15–20 ml h⁻¹. The absorption at 260 nm of each fraction (4.5 ml) was determined and hence the amounts of adenine and guanine were calculated. The samples were dried and counted for radioactivity after addition of water (1.3 ml) and Triton-toluene phosphor (10 ml). These measurements were used to estimate the amounts of methylated purines.

* Amounts of alkylation products are given as μmol methyl per mol DNAP.

appearance. This included the majority of animals (see ref. 7) and the remainder, which had sparsely nodular livers, are included in a separate study. Measurements of the exhalation of radioactively labelled CO₂ (ref. 8) indicated that the rate of metabolism of (^{14}C -methyl)-labelled DMN was similar in both control and AAF-pretreated rats. In agreement with previous reports for normal rats⁸ the lower doses were metabolised more quickly but the process was nevertheless complete by 5 h in all cases. At this time the level of 7-methylguanine was similar in liver DNA from control and AAF-pretreated rats (Fig. 2b) and in both cases the amount formed was linearly related to dose. The amounts of O^6 -methylguanine at 5 h were also dose-related but they were significantly lower for the AAF-pretreated rats, indicating that at all dosages the repair of this product was already well advanced (Fig. 2a): A comparison of the relative persistence of this base over a 48 h period further emphasised the differences observed between the two treatment conditions (Fig. 3). Thus, in control animals 5 h after DMN treatment at doses of 5.5 or 9 mg kg⁻¹ this ratio of O^6 -methyl- to 7-methylguanine approximates to the expected value of 0.115 for DMN⁶. At the highest dose there was a slight increase in this ratio over the next 20 h or so (Fig. 3a) and this rise can be accounted for by calculation of the expected loss of 7-methylguanine by chemical depurination, indicating that the amount of O^6 -methylguanine had remained constant for this period. After this, the ratio decreases rapidly so that by the 48 h the value approaches that obtained for the lower doses at later times. This inhibitory effect is much less apparent at 5.5 mg kg⁻¹ and was not detected at the 1 mg kg⁻¹ dose. Also, at 1 mg kg⁻¹ the ratio of methylated guanines is much lower, indicating that a greater relative proportion of the O^6 -methylguanine initially present in DNA (see Figs 2a and 3a) was repaired than at the high doses. A similar effect is seen in AAF-pretreated rats but at each dose level the repair of O^6 -methylguanine is enhanced, and at 9 mg kg⁻¹ this

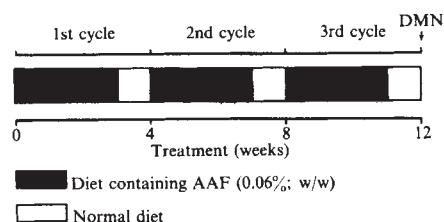


Fig. 1 Schematic representation of dietary regimen.

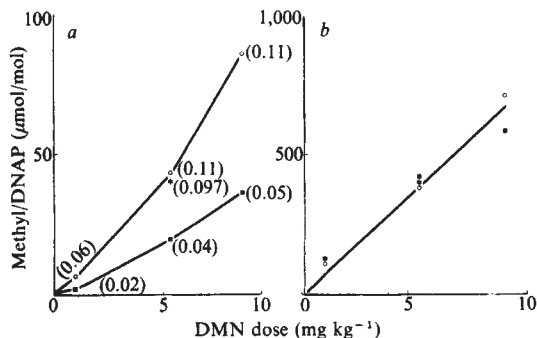


Fig. 2 Amounts of O^6 -methylguanine (a) and 7-methylguanine (b) in the liver DNA of control (○) and AAF-pretreated (■) rats 5 h after administration of (^{14}C -methyl)-labelled DMN at 1, 5.5 and 9 mg kg^{-1} (specific activity 11 mCi mmol^{-1} for lowest dose and 5 mCi mmol^{-1} for higher two doses). Liver DNA was prepared and analysed as previously described (see Table 1 legend) and the 5 h values are taken from lines of best fit for results obtained for animals killed over a 48 h period. Lines of best fit were derived as follows: a, quadratic curves ($P < 0.05$ in each case) for AAF-pretreated rats; Gompertz curves were fitted for the control data; b, least square regression lines (coefficient of multiple correlation $R > 0.98$ in each case). The ratios of the amounts of O^6 -methylguanine to 7-methylguanine are given in parentheses adjacent to the appropriate points. Values of these methyl purines taken from Table 1.

occurs to such an extent that there is no detectable inhibition of the repair process at all (Fig. 3b). Some recent reports have shown that chronic treatment with agents which produce O^6 -methylguanine in DNA can also induce the repair of these lesions. This has been observed for rat liver DNA following chronic administration of DMN⁹, in several organs of the rat following repeated injections of dimethylphenyltriazen¹⁰ and for *Escherichia coli* following pretreatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine¹¹.

In the AAF-pretreated animals the reason for the observed increase in the ratio of methylpurines at around 8 h (Fig. 3b) is obscure, but as metabolism of DMN is complete by the fifth hour no further increase in the amount of alkylation at the O^6 -position of guanine could occur. Further, as reincorporation of this product is extremely unlikely¹², one possible explanation might be the induction of a specifically enhanced loss of 7-methylguanine over this short period: such a process has not yet been observed for rat liver DNA but this could be a side effect of the stimulation of the system for the repair of O^6 -methylguanine in AAF-pretreated rats. For comparison, evidence for the repair

of 7-methylguanine has been given in certain cases, for example in the DNA of the livers of mice¹³ and of Syrian hamsters¹⁴.

In conclusion, we have observed a dose-related repair of O^6 -methylguanine in normal rats which, in agreement with previous work^{6,15}, is relatively more active at lower doses of DMN. Furthermore, a similar repair process is also present in AAF-pretreated rats, but here the repair of O^6 -methylguanine is significantly enhanced at all doses used (Fig. 3). This effect is particularly evident at early times and when compared to controls there is a two- to threefold decrease in the ratio of these methylated guanines. It is interesting that a prolonged exposure to one carcinogen can induce the repair of lesions formed in DNA by another chemically unrelated carcinogen. Such an effect might be due to the induction of a general repair mechanism, possibly analogous to the induction of post-replication repair observed in AAF-treated Chinese hamster cells¹⁶. Alternatively, this might also be explained by the activation of a more specific repair system due to the formation of small amounts of an AAF-adduct at the O^6 -position of guanine in DNA, particularly as such lesions have now been reported for the reaction of another aromatic amine, 1-naphthylamine, with DNA¹⁷. The formation of a lesion at a common site and its subsequent control by similar or closely related repair systems, which is implicit in the latter interpretation, may be especially relevant to the concept of mechanisms of carcinogenesis arising from agents belonging to different classes of chemical carcinogens.

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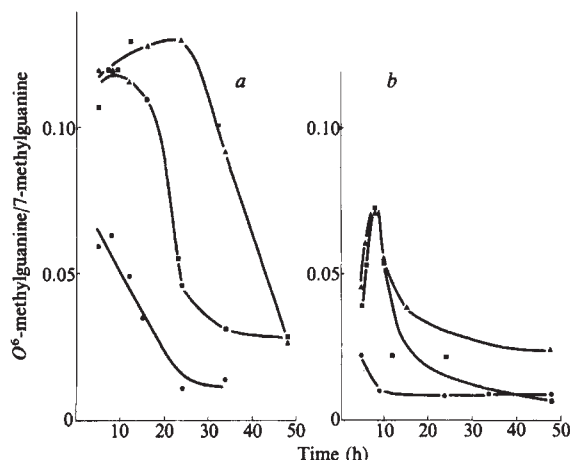


Fig. 3 Ratio of O^6 -methyl- to 7-methylguanine in liver DNA in control (a) and AAF-penetrated (b) rats at various times after injection (i.p.) of (^{14}C -methyl) labelled DMN at 9 mg kg^{-1} (▲), 5.5 mg kg^{-1} (■) and 1 mg kg^{-1} (●).

Corrigendum

In the letter 'Use of Panamanian sea urchins to test the molecular clock' by H. A. Lessios, *Nature* **280**, 599–601, line 4 in paragraph 6 should read '... in *Diameda* were 20 times higher than in *Echinometra*, we would ...'

Erratum

In the letter 'Induction of sporulation in *Bacillus brevis* by peptide antibiotics' by H. Ristow *et al.*, *Nature* **280**, 165–166, in paragraph 2 line 6, for "... that of gramicidin D between t_1 and t_5 ," read "... that of gramicidin D between t_1 and $t_{1.5}$." Also for the abscissa on the top of Fig. 1 " $t_1 \dots t_7$ ", read " $t_0, t_1 \dots t_6$ ".

matters arising

The cosmological evolution of QSOs

RECENTLY, Stewart and Hawkins¹ claimed that what is usually considered strong evidence for cosmological evolution of the number density of quasi-stellar objects (QSOs) is merely a statistical sampling effect. They find that the assumed evolution law, combined with the optical luminosity function of QSOs, predicts results that conflict with the observational data from which these two functions are derived. However, their result is due to several incorrect assumptions, and I suggest there is no such conflict for the largest homogeneous QSO sample available. Cosmological redshifts are assumed here throughout.

The proposal that the observed upper envelope of the points in the redshift-absolute optical luminosity plane is the result of statistical sampling¹ is not new. Schmidt² introduced the V/V_m test partly to remove this obvious selection effect, and a related method that also avoids the problem was proposed by Lynden-Bell³. In the V/V_m test, the number density evolution law is derived such that the selection effect is taken into account, and the luminosity function is then derived for a particular epoch (redshift) by applying this density law to the observed number densities at other redshifts represented in the sample. Note that the luminosity function cannot be derived independently of the assumed density evolution law. Stewart and Hawkins¹ claim that the upper envelope of the points in the $\log F_{2500}$, $\log z$ plane is satisfactorily reproduced only if there is no significant density evolution, and that if the previously-derived evolution law and luminosity function are assumed there is a serious conflict between the predicted and observed envelopes (F_{2500} is the absolute luminosity, in W Hz^{-1} , at 2,500 Å rest wavelength, using a Hubble constant of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, and is related to Stewart and Hawkins absolute magnitude M_v by $\log F_{2500} = 13.32 - 0.4M_v$; z is the redshift). Apart from doubts concerning the use of the upper envelope as an efficient statistical indicator, I believe their calculations to be invalid for the following reasons: (1) if there is no density evolution, it is incorrect to adopt a luminosity function that was derived *via* the assumption of strong evolution; (2) if there is evolution, it is unjustifiable to extend the optical luminosity function to arbitrarily high values when there is clear

evidence³⁻⁶ for an upper limit; and (3) Stewart and Hawkins¹ have not taken radio selection effects into account properly. The probability that a radio-emitting QSO will find its way into a catalogue depends on its radio flux density, that is on its absolute radio luminosity and its redshift. This modifies their equation (8) for the number of QSOs detected as a function of z , by an amount that depends on the radio flux density limit. Those QSOs in Stewart and Hawkins¹ inhomogeneous sample (derived from ref. 7) that were originally found as radio sources come from surveys with quite different radio limits, which makes the modification of their equation (8) indeterminate.

Stewart and Hawkins¹ result therefore seems to be based on erroneous assumptions, and as a check I have calculated the expected distribution of points in the $\log F_{2500}$, $\log z$ plane for the largest homogeneous QSO sample published so far⁵. This contains 74 QSOs identified as 4C radio sources, with stringent radio and optical flux density limits. Redshifts, and therefore values of F_{2500} , are known for 71 of them⁸. It has been shown⁵ that the luminosity function for the objects is adequately represented by:

$$dN \propto F_{500}^{-2.65} F_{2500}^{-1.08} V^{1.55} \times dF_{500} dF_{2500} dV \quad (1)$$

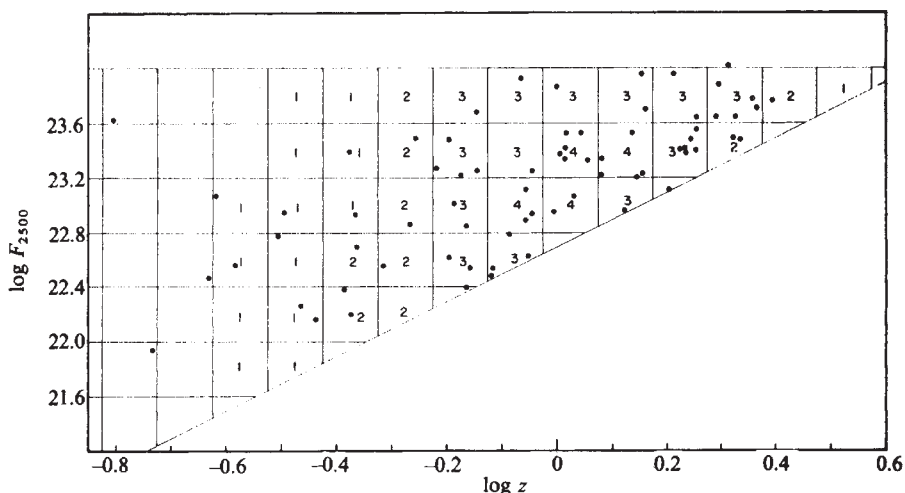


Fig. 1 Relationship between absolute optical luminosity (F_{2500}) and redshift (z) for QSOs in the homogeneous sample (from ref. 5). The predicted distribution is shown as the numbers of QSOs in cells 0.4 wide in $\log F_{2500}$ and 0.1 wide in $\log z$, or in the smaller areas if the cell is crossed by the sloping line, which is the limiting apparent magnitude of the sample. The predictions assume an upper limit of $10^{24} \text{ W Hz}^{-1}$ in the luminosity function³⁻⁶. Where no predicted number is given, it is <0.5 per cell. The total numbers of QSOs predicted in the intervals $\log z = -0.625$ to -0.725 , -0.725 to -0.825 , and <-0.825 are 2.0, 1.1 and 1.1 respectively. The points show the observed distribution.

carefully-compiled homogeneous samples of QSOs.

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STEWART AND HAWKINS REPLY—it seems clear that there is no essential disagreement between Wills' response and the primary result of our original paper¹; that if one takes the evolutionary law and luminosity function for quasars widely found in the literature and models the (L, z) plot one finds a disagreement with the observations, whether one uses the large list of Burbidge *et al.*² or the complete sample of Wills and Lynds³, in the sense that there is a shortage of very luminous objects. The question now arises as to why this disagreement with the observations exists. Wills accepts that the evolution law and luminosity function are correct and solves the problem by involving a 'cutoff' in the luminosity of quasars, that is by saying that for some reason a quasar cannot be more luminous than 10^{24} W Hz⁻¹ at 2,500 Å.

An attempt was made¹ to discuss the distribution in the (L, z) plot without invoking a cutoff by relaxing some of the assumptions, in particular by allowing the evolution rate to be an adjustable parameter and maintaining the luminosity function unchanged, although allowing rather large error bars. Wills is not strictly correct in saying that the two cannot be distinguished as it is clear¹ that the slope of the upper envelope in the (L, z) diagram at low redshift is determined by the index of the luminosity function alone. The result¹ was that the distribution of quasars in (L, z) could be accounted for very naturally with low or zero evolution, and in particular the absence of very luminous objects was shown to be a simple geometrical effect, thus removing the need for a cutoff in quasar luminosity.

It is obvious that if one regards the results of previous measurements of quasar evolution as being beyond reproach one is forced to introduce a luminosity cutoff and all Wills' results follow. However, as this widely held position is based on the results of a single test—the V/V_m test—it seems to us that one ought to look more closely at this and

ask if it is really giving the right result. This question is treated more fully in another paper⁴.

If one insists that the V/V_m test is giving the right answer then Wills is quite right and a cutoff, evolution and so on are all required. However, given the results of ref. 1 it seems desirable to re-examine the V/V_m test, as it may be possible to abandon two *ad hoc* hypotheses, that is, quasar evolution and luminosity cutoff, if its results turn out to be incorrect.

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Mixed ligand chelate therapy for plutonium and cadmium poisoning

I HAVE accumulated information leading me to doubt the veracity of results published in a report¹ and a reply² of which I was the co-author. I have repeated the experiments personally, and thus far find that in the stated conditions¹ I do not confirm these results. The experiments reported for cadmium poisoned mice, for example, did not, contrary to data provided me, include experimental controls for the individual chelants at the stated doses. Further, treatment was given 5 min post-Cd and not 1 h, as stated. At the latter time no survival is observed. Neither can I confirm the results described for plutonium decorporation by diethylenetriaminepentaacetic acid/salicylic acid (DTPA/SA). In fact, from potentiometric titration studies which I now carry out as a screening method for the identification of mixed ligand chelate (MLC) systems potentially useful for biological applications, I find that the DTPA/SA systems do not form significant levels of MLCs with a cation similar to Pu(IV), namely Th(IV). However, DTPA and catechol or Tiron do form MLCs but that with EDTA instead of DTPA is superior.

I believe that MLC phenomena constitute a potentially superior approach for the treatment of metal poisons. However, the extent to which this is realised will be determined by the results of my own experiments and those of others. In the meantime, I am forced to conclude that the previously published results¹ are invalid.

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Mixed ligand chelation therapy

SCHUBERT AND DERR¹ have reported that mixed ligand chelates (MLCs) of DTPA and salicylic acid (SA) markedly enhance the clearance of ²³⁹Pu from mice. We have examined this procedure in rats (196–224-d-old females, HMT strain, from the MRC Radiobiology Unit, Harwell) using similar injection procedures and similar analytical procedures. Analysis for ²³⁹Pu in excreta from animals caged singly, six animals per group, as well as the kidneys, livers, spleens and carcasses, showed that there was no significant difference between either the DTPA group or the DTPA/SA group.

We have also investigated the extraction of ²³⁹Pu into heptane and found no evidence that Pu(EDTA)SA is lipophilic. Unfortunately the extraction procedure was not described; however, our extraction procedure has been used routinely to demonstrate the interaction of ²³⁹Pu⁴⁺ with phospholipids².

Furthermore, we have found that intravenously injected NaSA (2.0 mmol per kg) is acutely lethal to rats and it is known that 10–30 g of NaSA is lethal to man³; the injection procedure of Schubert and Derr would necessitate intravenous administration of 22 g of NaSA to man every 3 d.

After completing our work we learnt that Schubert had withdrawn some of his claims, but we still view his optimism for MLC therapy with caution. For MLC therapy to be successful it will require administration of a chelating agent sufficiently lipophilic to offset the hydrophilicity of the Pu DTPA complex. There is no reason to believe that such diverse species enter cells in synchrony; in addition, we might wonder if serum citrate could not be part of an MLC with Pu and DTPA. It is more likely that synergistic chelation therapy⁴ will contribute more to toxic metal decorporation than MLC therapy.

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Lowland Maya radiocarbon dates and the classic Maya collapse

SIDRYS AND BERGER¹ assert that "the demise of the stela cult and the breakdown of elite culture seem to have coincided" among the Late Classic Maya population of the Southern Lowlands. Their evidence leans heavily on a comparison of frequency distribution graphs for long count-dated Maya monuments and radiocarbon-dated organic materials. The authors claim that the graphs are "remarkably similar", and that their resemblance "helps to establish the reliability of the ¹⁴C frequency-distribution bar-graph method".

We feel that the radiocarbon data presented by Sidrys and Berger show such a bias in sampling that the conclusions they draw are unreliable and misleading. Of the 96 radiocarbon dates plotted for the 'elite contexts' (in contrast to 415 monument dates) 66 dates are from the site of Tikal—no other single site is represented by more than 5 dates. Since the authors are attempting to analyse a demographic phenomenon that took place over thousands of square kilometres and involved many population centres, we suggest that selecting nearly 70% of the samples from one site dangerously disregards basic tenets of sampling theory. To demonstrate the effect that such an extreme bias in the radiocarbon data can have on the interpretation made by the authors, we have replotted their frequency-distribution graph for elite contexts, specifically excluding the dates from Tikal. The dates from all of the other 26 sites and artefacts that are listed in their Table 1 have been left unchanged. For comparison we have also reproduced the authors' original graph, which includes the Tikal data.

The replotted data (Fig. 1b) fail to support any significant 'breakdown of

elite culture' during the eighth to eleventh centuries AD. It is not our intention to question whether or not a breakdown actually took place. Our point is that such a drastic difference in interpretation, based solely on the presence or absence of data from a single site, renders spurious Sidrys and Berger's argument that a connection between the demise of the stela cult and the breakdown of elite culture is supported by a similarity between the frequency distribution of monument dates and elite context radiocarbon dates.

While we endorse the trend towards increasing quantification of archaeological data, particularly for the purpose of hypothesis testing, we emphasise that studies involving demography must take into account the need for representative sampling of available sites and contexts.

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SIDRYS AND BERGER REPLY—Our study did take into account the need for representative sampling of available sites. The high proportion of Tikal cases in our ¹⁴C graph of elite contexts simply reflects the abnormally large number of aristocrats, priests and administrators present at Tikal—a factor that Sterns, Loria and Garber overlook. Population estimates for Classic Maya centres are difficult to obtain and their reliability may be off by at least a factor of two. Nonetheless, those

studying the Mayan culture are in general agreement that Classic period Tikal was the largest population centre, with about 50,000 inhabitants¹ (some estimates are as high as 80,000). The number of Tikal elite has been estimated² to be 1,000. In contrast the average population of the other 18 sites used in our Fig. 3a, according to our own 'guesstimates' based on house mounds and size of ceremonial area, as well as the little published demographic data available, falls somewhere between 3,000 and 9,000.

Our sampling procedure thus mirrors Classic Maya cultural reality—the overwhelming economic and political dominance of the capital city of Tikal. Viewed from this perspective, the above graph by Sterns, Loria, and Garber that eliminates Tikal from the total elite sample is the equivalent of a demographic study of the state of New York that does not include New York City. At the same time, we do emphatically reiterate that more Maya ¹⁴C dates from commoner contexts are urgently needed to resolve the issue of the Classic Maya collapse.

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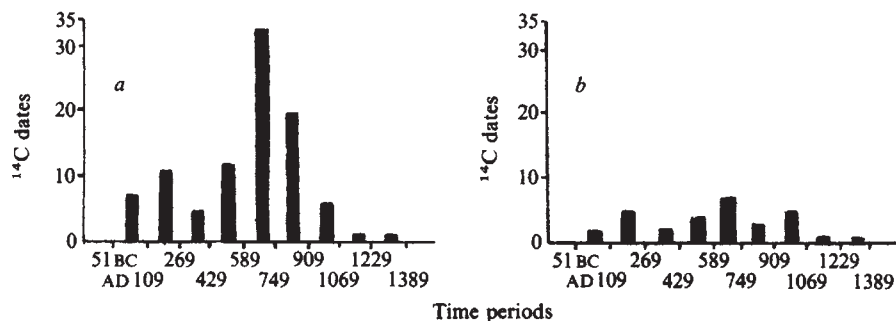


Fig. 1 a, Radiocarbon dates from elite contexts in the Southern Lowlands per time period, including dates from Tikal. b, The same data as in a, but excluding the dates from Tikal.

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Ca²⁺ and photoreceptor adaptation

FLAMING AND BROWN¹ seem to have missed the point of our earlier work on the effects of altered extracellular Ca²⁺ on rod photoreceptor responses², and they give a misleading impression of the possible role

of Ca^{2+} in mediating sensitivity changes that occur in rods during light and dark adaptation. We found that altering extracellular Ca^{2+} alters the amplitude of rod responses dramatically in the dark-adapted retina, but that the sensitivity of the rod (determined principally by σ , the position of the V -log I curve on the intensity axis) is virtually unchanged. As both amplitude and sensitivity vary substantially during light and dark adaptation, we concluded that changes in cytosol Ca^{2+} levels could not explain adaptation in photoreceptors. For example, we showed (Fig. 5 of ref. 2) that raising extracellular Ca^{2+} from 1.8 to 3.2 mM reduced $V_{\max}$ of the rod response by about 38%, but that in these conditions of high extracellular Ca^{2+} a significant shift in the position of the V -log I curve on the intensity axis could not be detected. There is, of course, some uncertainty in intracellular recording measurements, and therefore it is possible that within our error range (± 0.2 log units) some small changes did occur. However, such changes are insignificant compared to the changes that occur when a rod is adapted by a light that reduces its maximum amplitude response by the same amount that 3.2 mM Ca^{2+} reduces $V_{\max}$. For example, Fig. 6 of ref. 2 showed that an adapting light which reduced the rod response amplitude by 35% caused a shift of the V -log I curve along the intensity axis by at least 1.6 log units. In other words, in comparable conditions of $V_{\max}$ reduction, light causes a 65-fold change in rod sensitivity, but raised $[\text{Ca}^{2+}]_o$ changes sensitivity no more than 2–3 times. This was the main point on which our conclusion was based.

Flaming and Brown now report that if they raise or lower extracellular Ca^{2+} they can detect small changes in the position of the rod V -log I curve on the intensity axis and they suggest, therefore, that it is possible that alterations in cytosol Ca^{2+} levels can account for sensitivity changes in photoreceptors during light and dark adaptation. They report that raising extracellular Ca^{2+} from 1.8 to 6 mM causes the V -log I curve of the dark-adapted rod to shift by an average of 0.26 log units. In their Fig. 1 (ref. 1), they show that such an increase in $[\text{Ca}^{2+}]_o$ decreases a rod $V_{\max}$ by about 45%. If light is used to reduce $V_{\max}$ by this amount, however, the position of the V -log I curve alters not by 0.2–0.3 log units, but by about 2 log units. Thus, their data are in close agreement with our findings, and they support our conclusion that, "although cytosol Ca^{2+} concentrations are capable of regulating the membrane potential of the cell and its response amplitude, they affect only to a minor extent the sensitivity of the receptor cell in the dark-adapted or partially light-adapted state" (ref. 2).

Bastian and Fain³ have recently carried out experiments similar to those of

Flaming and Brown and ourselves. They found, in agreement with Flaming and Brown, that small shifts in the V -log I curve can be detected when extracellular Ca^{2+} is altered sufficiently, but their results also show clearly that the effects of increases in extracellular Ca^{2+} do not at all resemble the effects of background light on either rod sensitivity or response waveform. We may not have observed the small shifts of the V -log I curve in low and high Ca^{2+} because of the reasons outlined by Flaming and Brown. However, our failure to observe these changes in no way alters our conclusion that cytosol Ca^{2+} levels cannot be the major determinant of rod sensitivity during light and dark adaptation. Indeed, all three studies discussed here provide evidence in favour of this conclusion.

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FLAMING AND BROWN REPLY—in agreement with all other authors, Lipton *et al.*¹ found that increased extracellular Ca^{2+} markedly reduced $V_{\max}$. Their new finding was that raised or lowered extracellular Ca^{2+} concentration exerted no detectable effect upon σ , the stimulus intensity eliciting a response of half the maximum amplitude, although σ was altered significantly by adaptation. They stated that "The experiments do indicate that in a partially light-adapted rod the membrane potential and cytosol Ca^{2+} levels do not regulate the major determinant of sensitivity (σ), ...". We recently² showed that when external Ca^{2+} was varied from 0.3 to 6.0 mM, σ shifted to higher light intensities by about 0.75 log units. Both increases and decreases of Ca^{2+} concentration from the control level of 1.8 mM elicited consistent σ shifts that were detected readily. Our conclusions were: "Thus, our results indicate that alteration of $[\text{Ca}^{2+}]_o$ can control the value of σ in the V -log I curve of toad rods. These results are consistent with a role for $[\text{Ca}^{2+}]_i$ in the control of sensitivity in vertebrate photoreceptors. Hence, changes of $[\text{Ca}^{2+}]_i$ may partially control the changes of sensitivity that occur during light and dark adaptation". Our conclusion that $[\text{Ca}^{2+}]_o$ can exert a controlling effect on σ differs from the

conclusion of Lipton *et al.*¹ in an important respect, and we note that they now accept the validity of our findings on this point.

One remaining question concerns the degree to which $[\text{Ca}^{2+}]_i$ can control the value of σ . Taken at face value, our results suggest that $[\text{Ca}^{2+}]_i$ could only control a small portion of the adaptive σ shifts. However, we demonstrated that our Ca^{2+} -induced σ shifts depended on several improvements in experimental conditions, and one critical factor was a more normal perfusate containing 15 mM of bicarbonate, which was used for buffering² instead of HEPES¹. It thus seems that the induction of σ shifts by altering $[\text{Ca}^{2+}]_o$ is more sensitive to subtle chemical factors in the perfusate than are the effects of $[\text{Ca}^{2+}]_o$ on $V_{\max}$. This suggests that it is especially important to compare σ shifts induced by adaptation, and by alteration of $[\text{Ca}^{2+}]_o$, in physiologically normal experimental conditions. Although we have made some improvements in that direction, we are not convinced of having attained the requisite success, which may be very difficult with an isolated and perfused preparation. Also, even in a completely normal preparation, an elevated $[\text{Ca}^{2+}]_o$ may not adequately mimic the effects of increased $[\text{Ca}^{2+}]_i$. For reasons such as these, we deliberately drew no conclusion concerning the degree to which $[\text{Ca}^{2+}]_i$ may normally control adaptive σ shifts. Although Lipton *et al.*¹ failed to detect any of the controlling effect of $[\text{Ca}^{2+}]_o$ on the value of σ , their letter indicates great confidence concerning the quantitative control of σ by $[\text{Ca}^{2+}]_i$. As premature conclusions based on inadequate evidence can be 'misleading', it seems advisable to await more definitive evidence on this issue.

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reviews

Deficiencies in the Green Revolution

D.L. Gunn

Beyond the Green Revolution: The Ecology and Politics of Global Agricultural Development. By K.A. Dahlberg. Pp. 256. (Plenum: New York and London, 1979.) £11.30.

MILLIONS of people are undernourished or even starving, especially in the tropics. Yields of basic foods, generally cereals, are often poor. The Rockefeller Foundation therefore sent a team led by N.E. Borlaug into Mexico to breed more productive varieties of wheat for use in the tropics. He was very successful and the new seed has much increased crops in many places, for example enabling Pakistan to change from importing to exporting wheat. The technique has since been followed with other cereals. This has been called the Green Revolution, the great increase in food using scientific means.

Everyone involved in agriculture knows, however, that large increases in crop yields cannot be achieved and sustained by simply substituting the new seed for the old. More plant food (artificial fertiliser), extra water, herbicides and insecticides may all be required. Thus, full success with new wheat strains requires money to be invested in these extra inputs and also new knowledge and skills. The vast numbers of struggling subsistence farmers — about whom Dahlberg is mainly concerned — are too poor to use the new wheat.

Kenneth A. Dahlberg, a social scientist, has come across these well-known facts and, in this book, emphasising the deficiencies of the Green Revolution, he pleads for re-assessment of situations by "contextual analysis". For biologists there is nothing new in seeking to examine a situation as a whole before suggesting how to alter it; that is similar to examining a problem in its context. Dahlberg explores a very wide context. In three chapters entitled "Ecology of Theories", "Historical Seedbeds" and "Jack and the Beanstalk", he outlines the influence of European tradition, of climate on the kind of ecological theory produced, and of trade on economic thought; the resulting ideas have little relevance to subsistence agriculture in the tropics. Contextual analysis is put forward for examining situations instead of proposing actions merely because they would be effective in a temperate climate.

The remaining chapters are headed "The

Momentum of Structures", "Institutions and Current Policies"; "New Approaches to the Future"; "Agricultural Strategies and Policies for the Future"; and "Hindsight, Insight and Foresight".

The author is critical and censorious about the European colonial powers, especially the British, to whom he imputes selfish national motives. Actually many agricultural officers and district commissioners were more concerned about the welfare of the local people than about the trade interests of the mother country. They discovered most of the complications that Dahlberg expounds and their long and devoted service did help the peasants in many places; but they did not solve the problem of financing innovation — not merely a matter of providing the money. The British Government set up the College of Tropical Agriculture in Trinidad, through which intending agricultural officers passed. They were trained to understand both agricultural and sociological complications, either in the College or on the job.

Amongst the many criticisms of European influence, there is one commendation implied in the stated failure of North American charities "to draw upon the extensive experience of Europeans in semi-tropical research, extension and education"; but why semi-tropical?

There is of course no doubt that European influence in tropical countries has disrupted old ways, often regrettably; but should the destruction of the Ashanti, the Zulu and the Matabele bloodthirsty modes of life be regarded as good or bad? Those tribes certainly restricted the population growth of others; and the Zulus left much of the Transvaal empty and open to the incoming Boers.

Dahlberg does mention the explosive increase in human population as the most important change now taking place, but he merely asks "Can you condemn millions to starvation by not making every effort to increase agricultural production, especially if there is any chance that some solution to the population problem can be found in coming decades?" He hopes for options other than "lifeboat ethics".

It is this population increase in the less developed countries — and only there — that creates the problem. There would be little difficulty today in feeding the world

population of my youth, two thousand millions, but where will it end? Well-intentioned Christian missions, the pax Britannica, medico-sanitary innovations by Europeans in the tropics, saving the children, and famine relief in general, have been responsible for creating the problem and destroying long-term stability. The problem is not how to produce enough food for everyone at present, although that is how it is usually stated. The problem is how those who cannot produce enough for themselves are to pay others to produce it for them. It is a problem of poverty. What can the poor do or produce that the rest of the world will be willing to buy? Industrial development, rejected by Dahlberg, is one partial solution, although it increases the problem of urban poverty, constantly fed from a profligately breeding rural population. Producing enough food for everyone now, even if it has to be given away to the poor, merely postpones greater starvation. There is no remedy in the long term for food shortages and for losses of irreplaceable resources without stopping the growth of population.

The author cites the resistance of the developing countries at the 1972 Stockholm Conference to any interference by environmentalists (regarding, for example, use of pesticides) and interprets it as misplaced confidence by elitists in Western conceptions of development; it seemed to me to arise more from preference for preserving the lives of their people rather than their eagles. That was certainly expressed at the Royal Society Discussion on Control of Migrant Pests in 1977. He also refers to vested interests in the debate over pesticides, without mentioning specifically the large and lucrative anti-pesticide industry, which was prominent at Stockholm. He writes that the World Food Conference and other such planning meetings propose solutions to food supply problems that are anti-ecological, evidently using the word ecological in the modern propaganda sense rather than as it is used in biology. Indeed, his acceptance of alarmist propaganda makes one wonder how much reliance is to be placed in statements in less familiar spheres.

This book is well-intentioned, sometimes interesting, sometimes dreary, sometimes misleading, sometimes muddled — "What is needed are a series of policies..." and sometimes obscure —

"information forwarded by life...". A more objective and perhaps humble analysis of past searching for improvement, less censorious but critical where something could be learnt, would have been more acceptable. It would have been illuminating to have a comparison of progress in Africa with that in South America. The book may have something for social scientists; it has nothing for

agriculturalists.

There are nine pages of references and an index of 18 pages. In neither is Borlaug's name mentioned. □

D.L. Gunn, now retired, has been Director of the International Red Locust Control Service, Northern Rhodesia, Director of the Tea Research Institute, Ceylon, and Adviser to the Secretary of the Agricultural Research Council, London, UK.

African mammal palaeontology

Evolution of African Mammals. Edited by V. J. Maglio and H. B. S. Cooke. Pp.641. (Harvard University Press: London and Cambridge, Massachusetts, 1978.) £42; \$60.

POTENTIAL READERS should clearly appreciate that this volume represents the first effort to put together in one book complete overviews of all African mammal groups. I want to congratulate the editors, Maglio and Cooke, for providing the impetus which led each of many specialists to articulate for the first time the entire known African story of his group. It should be stressed that much of what is presented in this volume, both in terms of materials and discussion, is quite new.

Authoritative introductions on present-day mammals of Africa (Bigalke) and the geological development of the physical African setting (Cooke) set the stage for 27 systematic chapters. These vary greatly, and appropriately, in length and depth of treatment. There can be little doubt but that the 95 pages by Howell represent the most complete and useful treatment available of the African evolution of the family of man, the Hominidae. The paper combines some provocative interpretations with admirably balanced consideration not only of the different African fossil sources, but also of the legion of opinions expressed on and around hominid evolution.

Several other larger contributions are particularly impressive and comprehensive, notably those on Proboscidea (Coppens, Maglio, Madden and Beden), Bovidae (Gentry), Suidae and Tayassuidae (Cooke and Wilkinson) and Equidae (Churcher and Richardson). There are also substantial chapters on mesozoic mammals (Crompton and Jenkins), Insectivora and Chiroptera (Butler), Rodentia and Lagomorpha (Lavocat), prosimian primates (Walker), Cercopithecidae and Parapithecidae (Simons and Delson), cenozoic apes (Simons, Andrews and Pilbeam), *Ramapithecus* (Simons and Pilbeam), Carnivora (Savage), Pholidota and Tubulidentata (Patterson), Hyracoidea (Meyer), Deinotherioidea and Barytherioidea (Harris), Rhinocerotidae

(Hooijer), Anthracotheriidae (Black), Hippopotamidae (Coryndon), Cervidae and Palaeomerycidae (Hamilton), Giraffidae (Churcher), Sirenia (Domning) and Cetacea (Barnes and Mitchell).

Some shorter papers on lesser known groups add fascination by the strangeness of the beasts they describe: Embrithopoda (Tanner), Moeritherioidea (Coppens and Beden), Chalicotheriidae (Butler), Tragulidae and Camelidae (Gentry). Maglio's summary chapter on faunal turnover and succession, distribution and endemism, will probably be extensively used in future zoogeographic and evolutionary studies.

Much as each of the 30 fine contributions in this volume deserves individual comment, in a review of this length I will have to be content with expressing some overall impressions. The chronology, geography and stratigraphy of fossil finds are exceptionally completely and lucidly covered in numerous tables, maps and discussions. The histories of early fossil finds and conclusions are full of interest, and occasional humour. For example, in Harris' work on deinotheres we are told: "Buckland's (1835) quaint portrait of a mammal anchored to a river bank by its tusks while it slept preceded the discovery of deinother hind limbs". Illustrations pinpointing precisely salient morphological differences or primitive-to-advanced series, like those provided by Gentry (p544), Crompton and Jenkins (pp45 and 50), Hamilton (p506) and others, are valuable in a reference book of this sort. It is regrettable that the book contains too few of them.

Bibliographical coverage is excellent, with only a few exceptions (for example, the lack of reference to White and Harris' (1977) major work on fossil suids in the chapter by Cooke and Wilkinson; omission of several papers on African Carnivora, for example, of M.G. Leakey, Howell and Pether; and only cursory reference to important contributions by other carnivore specialists, such as Hendey and Ewer, in the contribution by Savage; and because the phylogenetic status of *Ramapithecus* is by no means established, as Simons and Pilbeam readily admit in their paper on this taxon, the inclusion of a larger bibliography and debate pro and contra hominid status of *Ramapithecus* would have further added to the great interest of this chapter).

In several instances good reviews of past and ongoing debates make exciting reading, as in the discussion of cetacean origins and the phylogenetic position of the African Archaeoceti by Barnes and Mitchell. Fortunately for a palaeontological tour de force of this length several of the authors have the ability to bring their long-dead creatures to life again. The expression of such ability, from deliberations on brain size and locomotion in the earliest mammals (Crompton and Jenkins) and on the evolution of the ant-eating habit in aardvarks (Patterson), to questions about how deinotheres used their tusks (Harris) and about why sirenians speciate slowly (Domning), adds greatly to a real enjoyment of this book.

In the preface the editors remark: "Such overviews (as this one) are the only means of . . . picking out our significant achievements and failures". On reading through the whole book others too may perceive, as I do, a disturbing failure common to a sizeable block of the present contributions. On the one hand many morphological details are given; on the other, conclusions on relationship (in some cases 'trees') are presented. But between these two there is often an hiatus; that is, it is difficult, at best, to extract precisely which morphological details are considered by the author pro, and which contra, his hypotheses of relationship. The reader is thus unable to test such hypotheses. In individual cases it is not clear whether the author has preferred to base his conclusions only on unsorted impressions; or whether he *has* used a particular methodology to arrive at clear statements supporting his conclusions, but has merely failed to articulate these statements to his readers. Several of the papers stress the paucity and fragmentary nature of the fossils. That such shortcomings do not justify a lack of methodology and clarity is shown by the fact that some contributors on the poorest fossil samples (for example, Crompton and Jenkins on Mesozoic mammals, Hamilton on Cervidae and Palaeomerycidae, Patterson on Pholidota and Tubulidentata, and others) are among those that make the clearest statements in support of their hypotheses of relationship.

This large, sturdy volume, in which printing, reproduction of illustrations, binding and indexing are all of high quality, is unique in fully representing present knowledge of African fossil mammals. Whether or not one's own interpretations (and indeed mode of interpretation) differ from any of those in *Evolution of African Mammals*, there is no better companion than this book for anyone, whether student or specialist, who is interested in African mammal palaeontology.

E.S. Vrba

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Plastic crystals

The Plastically Crystalline State: Orientationally-Disordered Crystals. Edited by John N. Sherwood. Pp. 383. (Wiley: Chichester, UK, and New York, 1979.) £19.50.

THIS is a valuable and interesting book, dealing with a type of crystal to which increasing attention has been paid in recent years. Plastic crystals are frequently rather arbitrarily defined as those for which the entropy of fusion is less than $\sim 20 \text{ J K}^{-1} \text{ mol}^{-1}$, but the editor wisely decided that his contributors should not be rigidly confined by this limitation, and references are made to orientationally disordered solids not satisfying this entropy of fusion criterion, and occasionally also to salts and to liquid crystals.

The book is essentially a collection of essays by experts on some of the techniques available for investigating orientational disorder in crystals, and especially of newer methods still in the evolutionary stage. There are no accounts of certain techniques which, though admittedly of longer standing, will nevertheless continue to be used, such as experimental thermodynamics and neutron diffraction. The topics covered include methods of studying self-diffusion, diffuse X-ray scattering, dielectric and acoustic studies, NMR, Rayleigh and Brillouin scattering,

infrared and Raman spectroscopy, and neutron scattering. There are also chapters on the structures of plastic crystals, on the correlation of dynamic observations of molecular motion, and on the theoretical aspects of orientational disorder. The quality of exposition is generally high, and the text is illustrated by well chosen figures.

A typical chapter describes the theory and practice of the technique with which it is concerned, and there then follow examples of its application to particular systems. In consequence, the sum total of the space devoted to any particular crystal can vary considerably. Thus, there are 29 references in the index to adamantane, 35 to cyclohexane, and 23 to hexamethylethane, which in each case are distributed fairly uniformly throughout the book. On the other hand, there is very little on the plastic crystalline phases of nitrogen, oxygen, carbon monoxide and fluorine, even though these have received a good deal of attention on account of their comparative molecular simplicity.

When this book has so much to commend it, it is a pity to have to add that it contains more minor misprints and mistakes than one might have expected to find. However, these are minor blemishes on a book which should be a real asset to those actively interested in the subject.

L.A.K. Staveley

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Control of movement and posture

An Introduction to Physiology. Vol.4. By H. Davson and M.B. Segal. Pp.573. (Academic: London; Grune and Stratton: New York, 1978). £11.80.

THE latest volume in this impressive series is devoted to the control of movement and posture, which it treats in considerable depth. The first section begins with an instructive general account of quadrupedal locomotion which, as much experimental work is performed on four-footed subjects, is clearly justifiable on scientific grounds, but may not be thought wholly relevant by readers primarily interested in man.

This is followed by a brief discussion of the grading of muscular effort and a detailed description of proprioceptive sensors leading on to a consideration of spinal reflexes, which starts from the work of D.P.C. Lloyd in 1943 rather than, as might have been expected, from that of Sherrington. In this chapter the contributions of the Matthews, father and son, to spindle-physiology, together with

modern views on the significance of the tendon organs, are surveyed.

The second section of the book is concerned with supra-spinal control of motor activity and contains chapters on the vestibular apparatus, cerebral cortex, cerebellum, reticular, olivary and red nuclei, and also the basal ganglia. The final chapter comes full circle and returns to broader aspects of the coordination of movement. In dealing with topics as complex as these it is immensely difficult to create for the first-time reader (for whom, presumably, an 'introduction' is intended) a clear and coherent outline but a brave effort has been made and, on the whole, successfully.

The text is conveniently subdivided by frequent and informative headings and is copiously illustrated and documented with references. Most of the illustrations display the experimental results obtained by the investigators whose work is discussed while the remainder are explanatory diagrams.

My assessment of the status of this multi-volume treatise was incorporated in the review of an earlier volume see (*Nature* 266, 284; 1977) and remains unchanged.

R.V. Coxon

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Paper £7.95



Edward Arnold

41 Bedford Square,
London WC1 3DQ

Stellar evolution

Stars and Clusters. By Cecilia Payne-Gaposchkin. Pp. 261. (Harvard University Press: Cambridge, Massachusetts, and London, 1979.) £13.50; \$22.50.

PAYNE-GAPOSKIN was in no small way responsible for my deviation from the straight and narrow path of pure physics to the ethereal delights of astronomy. I devoured her book *Introduction to Astronomy* (Eyre and Spottiswoode, 1956) during my second year at university and never looked back. That book, like *Stars and Clusters*, is intended for the student and the general reader who may have little background in mathematics or physics. To this end *Stars and Clusters* is written without recourse to any mathematical formula; not a single equation graces its pages. Our innumerate unphysical friend can therefore romp through the text with ease.

But what about the figures? I was immediately struck by the fact that the figures must be considerably more difficult to understand than the text if you didn't 'have the mathematics'. The intricacies of the Hertzsprung-Russell diagram especially when it contains multiple stellar evolutionary tracks are difficult enough to grasp at the best of times. Our non-mathematical student has probably never drawn a graph in his life, has no idea what a logarithm is and must be confused by the unexplained leap from $(B-V)$ to log surface temperature for the abscissa without the added complication that the later scale is inverted. It is highly laudable to write a book with the non-mathematical student in mind, but if this student is to gain most from the book I think he must be taught some maths on the way; he must, to quote Martin Johnson, 'discover for himself how little armoury he needs for appreciating the beauty and conviction of tracing an argument in symbols and actual quantities'.

Stars and Clusters falls between two stools. The innumerate student would appreciate help with the graphical concepts and the numerate student would benefit greatly from a mathematical treatment. This of course isn't to say that I did not enjoy reading the book. I did. But I was constantly reminded that one small equation must be replaced by many and complex words if the same message is to be conveyed.

The main theme of the book is the effect of evolution on stellar characteristics, the physical processes underlying stellar evolution and the light thrown on to this problem by the observation of open clusters, globular clusters and variable stars. The book is impressively illustrated and it is obvious that enormous care has been taken in the choice of the figures. An

indepth study of the Hyades cluster in Taurus is used to introduce the early stages of stellar evolution, and one of the figures is a plot of apparent visual magnitude as a function of $(B-V)$ colour for all the stars in the Hyades field of view. The interpretation of this figure would be a fascinating problem for a first-year astronomy student at university. The same can be said of a figure in the next chapter, the path of a metal rich star of five solar masses on the Hertzsprung-Russell diagram. I have the sneaking suspicion that both these would leave the amateur astronomer way behind. The book contains about thirty figures showing photographs of clusters and absolute magnitude against colour plots of their stars; again a fascinating study for the university student. A careful distinction is made between the evolution of single stars and the peculiar mutual influence that double stars exert on each other and on their evolution. The book contains a superb collection of light curves for variable stars;

one indication of its thoroughness is that there are 48 for R.R. Lyrae stars alone! The AAVSO star atlas is included at the end of the book so if you want to find, for example, X Geminorum you are shown where to look.

I am going to recommend this book very strongly to my first-year university students. They will be able to understand the figures, and will not need all the footnotes that rather verbosely explain the simple physical concepts. It may seem strange but the fact that it contains no mathematics makes me reluctant to recommend it to an innumerate student. Many of the concepts introduced and most of the concepts illustrated need a more detailed and also a more numerate explanation to make them understandable.

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Conidium ontogeny

Patterns of Development in Conidial Fungi. By G.T. Cole and R.A. Samson. Pp. 190. (Pitman: London, San Francisco and Melbourne, 1979.) £29.75.

SINCE S.J. Hughes in 1953 firmly placed developmental characteristics of conidial fungi amongst the prime ones for classifying Fungi Imperfecti, studies of conidium and conidiophore ontogeny have galloped apace. There is no doubt that these researches have generated abundant new taxonomic criteria or have illumined established ones. The difficulty has largely been in judging the significance of these characteristics. The fact that a number of attempts have been made to erect more or less formal classifications based primarily on features of conidiophore and conidium development shows that conidial fungi undoubtedly can be classified by means of these criteria, but what is less clear is whether such classifications reflect the phylogeny of the organisms concerned.

It is because the taxonomic significance of ontogenetic data is still an area of lively controversy that this new book is such a timely and valuable contribution. The authors have brought together an enormous amount of information, chiefly in pictorial form and much of it original (mainly as new scanning electron micrographs), so that the theorising mycologist who wishes to reclassify the fungi and the morphogeneticist who wants to find how fungi come to look the way they do have very little need to look at real live fungi—at least, while they are planning how best to go about their studies. The quantity of factual information is so large and the authors' presentation so good that

virtually all that is needed for armchair speculation is available here in a single convenient volume. The authors themselves are reticent about the formal taxonomic implications of the book's contents. They are explicitly and unambiguously concerned with patterns of conidial development, and it is these which they classify, not the fungi themselves.

Two-thirds of the book deals with various modes of conidium ontogeny in Fungi Imperfecti, chiefly hyphomycetes. The remainder includes an account of techniques for studying the development and ultrastructure of conidial fungi. Because so many of the electron micrographs presented are original it was wise of the authors to have included this chapter which effectively amounts to a 'materials and methods' section. There is also a major section on patterns of sporangiospore development in Zygomycetes which serves to illustrate how another group of mycelial organisms has solved problems similar to those facing higher fungi. A glossary eases the way for those not familiar with the subject's specialised terminology.

The text is copiously and beautifully illustrated and astonishingly free from printing errors. The authors write well (that is, they say precisely what they mean), and though they use specialised terms where appropriate, they eschew jargon. The high quality of the book's format is reflected in its price, but despite this it is likely to appear in most mycology teaching libraries, as it furnishes in convenient form so many data and concepts. Researchers in the field of fungal taxonomy and developmental biology will use it for these reasons and as a source of esoteric pleasure.

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